HIV pathogenesis in the female genital tract during chronic HIV infection: The impact of inflammation, T cell memory differentiation status and homeostatic cytokines on mucosal T cell immunity

by

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This dissertation is dedicated to my parents

Allison and Thobile Mkhize

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LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
ARV	Antiretroviral drug
APC	Allophycocyanin
APCs	Antigen presenting cells
CCR4	Chemokine receptor 4
CCR5	Chemokine receptor 5
CCR7	Chemokine receptor 7
CD	Cluster of differentiation
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
CXC	Cys-X-Cys
CVL	Cervicovaginal lavage
DCs	Dendritic cells
DC-SIGN	Dendritic cell-specific-Inter-cellular adhesion molecule 3-grabbing non integrin
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EBV	Epstein Barr virus
ELISA	Enzyme-linked immunosorbent assay
Env	Envelope glycoprotein
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothyocyanate
FMO	Fluorescence minus one
FSC	Forward scatter
Gag	Group specific antigen
GALT	Gut-associated lymphoid tissue
HAART	Highly active antiretroviral therapy
HEPS	Highly exposed persistently seronegative
HIV-1	Human Immunodeficiency type 1
HSV-2	Herpes simplex virus type 2
h	Hour
ICS	Intracellular cytokine staining

IL	Interleukin
IQR	Interquartile range
LTNP	Long-term nonprogressor
MALT	Mucosa-associated lymphoid tissue
MAP	mitogen activated protein
MHC	Major Histocompatibility Complex
MIP-1β	Macrophage inflammatory protein 1 beta
ml	Millilitre
NF-κB	Nuclear factor kappa B
PBMC s	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PE	Phycoerythrin
PerCP-Cy5.5	Peridininchlorophyll protein-Cy5.5
PMA	Phorbol myristate acetate
RANTES	Regulated upon Activation, Normal T cell Expressed, and Secreted
RNA	Ribonucleic acid
RPMI	Rosewell Park Memoral Institute Medium
SEB	Stapylococcal enterotoxin B
SHIV	Simian Human Immunodeficiency Virus
SIV	Simian Immunodeficiency Virus
SSC	Side scatter
STI	Sexually transmitted infection
TCR	T cell receptor
TNF- α	Tumour necrosis factor alpha
USA	United States of America
Vpr	Viral protein R
µg	Microgram
µl	Microlitre
^{0}C	Degrees Celsius

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Abstract

Background. The female genital tract serves as the major portal of entry for human immunodeficiency virus (HIV). Local immune factors unique to the mucosal micro-environment such as the genital tract cytokine milieu or the activation/differentiation status of T cells may play a significant role in heterosexual transmission of HIV and subsequent pathogenesis. Elucidation of the mechanisms underlying the persistent recruitment, activation and differentiation of mucosal T cells will give crucial insight into potential therapeutic targets to restore effective local immunity. The aims of this study were to (1) investigate the relationship between HIV-specific CD8 T cell responses in the female genital tract, local control of genital HIV shedding and genital inflammation in HIV-infected women; (2) investigate the impact of HIV infection and disease status on the memory phenotype and functional ability of T cells derived from female genital tract compared to blood; and (3) evaluate the role of inflammatory and homeostatic cytokines in the genital tract on the differentiation status of cervical T cells and maintenance of mucosal CD4 T cells.

Methods. Cervical cytobrushes and blood was collected from a total of 60 uninfected and 63 HIV-infected women. Phenotypic (CD3, CD4, CD8), maturational (CD45RA, CD27 and CCR7) and functional (IFN-γ and IL-2) markers were measured on cervical and blood-derived T cells by intracellular cytokine staining flow cytometry in response to stimulation with HIV Gag (Du422 subtype C) overlapping peptides or PMA/ionomycin. Genital tract and blood concentrations of inflammatory and homeostatic cytokines were measured by Cytometric Bead Array (TNF-α, IL-10, IL-1β, IL-6, IL-8, IL-12p70) or high sensitivity ELISAs (IL-7 and IL-15). HIV RNA copies in plasma and genital secretions were measured and compared to inflammation and HIV-specific T cell immunity in the female genital tract and in blood.

Results. This study shows that the frequency of CD4 T cells in both the female genital tract and blood was significantly reduced in HIV-infected compared to uninfected women and that the extent of CD4 depletion correlated significantly between compartments. Irrespective of HIV status, reduced frequencies of CD4 T cells at the cervix were associated with reduced frequencies of CD4 and CD8 T cells in the early stages of differentiation (central memory) in the genital tract, but elevated frequencies of cervical T cells in the late stages of differentiation (intermediate and terminally-differentiated effector CD4 and CD8 T cells). Genital tract concentrations of IL-7 were found to be negatively associated with the extent of cervical CD4 T cell depletion, irrespective of HIV status, suggesting that local IL-7 concentrations increase in

response to depletion of cervical CD4 T cells. Effector memory T cells were the most dominant T cell memory subset isolated by cervical cytobrush, irrespective of HIV status or the extent of CD4 depletion. This study shows that HIV Gag-specific CD8 and CD4 T cell IFN-γ (and to a lesser extent IL-2) responses were detectable in both the cervical and blood compartments of HIV-infected women and were of a significantly higher magnitude than responses similarly measured in uninfected women. The net magnitudes of CD8 T cell IFN-γ responses to HIV-Gag in the cervical compartment were not associated with the corresponding magnitude of IFN-γ responses in blood, indicating that *ex vivo* HIV responses in blood were not predictive of those in the female genital tract. A significant positive association was observed between the magnitude of cervical HIV-specific CD8 T cell responses and the concentrations of inflammatory cytokines in cervical supernatants. Compared to the CD8 T cell compartment, few HIV-infected women had HIV-specific CD4 T cells at the cervix, with IL-2 responses being more readily detected than IFN-γ. In addition, in response to PMA/ionomycin stimulation, this study found that significantly lower frequencies of genital tract effector memory CD4 T cells from HIV-infected women produced IFN-γ and IL-2 than uninfected women suggesting that CD4 T cells from the female genital tract may be functionally impaired during HIV-infection. Twenty-eight of the 63 (44%) HIV-infected women included in this study were shedding HIV in their genital secretions. No association was found between the magnitude of cervical HIV-specific CD8 T cell responses and mucosal HIV shedding suggesting that CD8 T cells present in the genital tract were not providing protection against genital HIV shedding. The concentrations of both inflammatory (IL-1β) and homeostatic (IL-7) cytokines were significantly higher in genital secretions from HIV-infected women compared to uninfected women. In addition, women who were found to be shedding HIV at the genital tract had significantly elevated concentrations of genital TNF-α, IL-1β, IL-6 and IL-8 compared to women who were not shedding HIV.

Conclusions. This study showed that inflammatory cytokines in the female genital tract may promote HIV shedding possibly by recruiting more targets (including HIV-specific T cells) to serve as targets for ongoing HIV replication. HIV-specific cervical CD8 T cells that secreted IFN-γ were not able to control HIV shedding. CD4 T cell depletion in the female genital tract was associated with the accumulation of mature T cell phenotypes and genital IL-7 was found to be involved in the homeostatic regulation of cervical CD4 T cells. These studies show that inflammation is likely to be important in HIV transmission. Furthermore, this study suggests that restoration of memory T cells by homeostatic cytokines (IL-7) in HIV infection may only be feasible when inflammation (activation) and the inevitable activation-induced cell death are controlled.

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Chapter 1
Literature Review

1.1 Introduction

Since the discovery of human immunodeficiency virus (HIV) in 1983 (Barre-Sinoussi *et al*., 1983), HIV has infected approximately 60 million people world-wide, and has already claimed more than 25 million lives (UNAIDS, 2008). Sub-Saharan Africa remains the region most heavily affected by HIV worldwide (Figure 1.1, UNAIDS 2008; 2010). Over two thirds (67%) of all people living with HIV are in Sub-Saharan Africa, accounting for nearly three quarters (72%) of AIDS-related deaths in 2008 (UNAIDS, 2010). South Africa continues to be home to the world's largest population of people living with HIV with an estimate of 5.7 million people in 2007 (UNAIDS, 2010). HIV is the leading cause of death and disease among women of reproductive age (15-49 years) worldwide (UNAIDS, 2010). Women appear to be at higher risk for infection than men, with an estimated 30% to 40% of annual worldwide infections occurring through HIV invasion of the female genital tract via exposure to virus-containing semen (Hladik and Hope, 2009). In sub-Saharan Africa, 60% of the people living with HIV are female. A disproportionate number of young women aged 15-24 years in Southern Africa are infected with HIV, with levels of infection about three times higher than among men of the same age (UNAIDS, 2010).

Although remarkable progress has been made in reducing HIV-associated morbidity and mortality through the development of a large repertoire of anti-retroviral drugs, we still do not have a cure for HIV infection and an effective vaccine has not been successfully designed (Sodora and Silvestri, 2010). This is largely due to the fact that HIV is more complex than other human viruses owing to its extraordinary mutability and resulting genetic diversity that leaves the immune system defeated. Since women are the most vulnerable to HIV infection (Hladik and Hope, 2009), it is important to understand the pathogenesis of HIV infection in women particularly in the female genital tract where the virus is initially encountered with the goal of identifying prevention strategies targeting transmission from HIV-infected women. This thesis

focuses on understanding poorly defined mechanisms involved in HIV pathogenesis within the mucosal cervicovaginal area in women chronically infected with HIV-1.

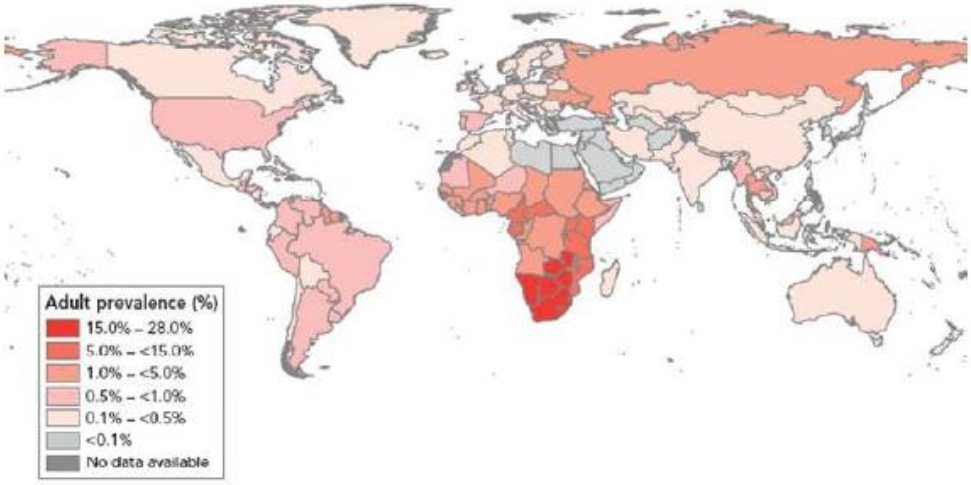

Figure 1.1 Adult prevalence of HIV infection in Sub-Saharan Africa compared to other geographic regions. The darker colours indicate regions with the highest prevalence whereas the lighter colours indicate regions with the lowest prevalence. Sub-Saharan Africa is the most impacted region (adapted from UNAIDS, 2008).

1.2 The mucosal immune system

The mucosal surfaces of the respiratory, gastrointestinal and reproductive tracts serve as the major portal of entry for invading pathogens making them constantly exposed to foreign antigens (Ganz, 2002; Mowat, 2003; Nagler-Anderson, 2006). The mucosal immune system must be able to combat invading antigens while also inducing tolerance to commensal microbes that do not require an immune response (Mowat, 2003; Holmgren and Czerkinsky, 2005; Magalhaes *et al.*, 2007). The respiratory, gastrointestinal and reproductive tracts are equipped with a broad range of elements of innate and adaptive immunity (Shacklett *et al.*, 2009). Epithelial cells which serve as mechanical barrier to pathogen entry in all mucosal surfaces are able to secrete cytokines, growth factors, chemokines, antimicrobial peptides and present antigens (Pitman and Blumberg, 2000; Shacklett *et al.*, 2009). The respiratory and gastrointestinal tracts contain organized mucosal-associated lymphoid tissue (MALT). In the gut, adaptive immune responses take place in gut-associated lymphoid tissues

(GALT), Peyer's patches and lymph nodes where antigen is taken up and redirected towards priming of T and B cell responses whereas the epithelial lamina propria serves as a site of antigen sampling (Mowat and Viney, 1997; Mowat, 2003).

Although the genital tract has been suggested to contain inducible MALT aggregates consisting of T and B cells (Brandtzaeg, 1997; Rier and Yeaman, 1997; Johansson *et al*. 1998), there is no evidence for the organized lymphoid structures at this site. The genital tract is a tertiary effector site (Wu *et al*., 2000; Russell and Mestecky, 2002) and immune cells residing here are thought to be recruited in response to an inflammatory signal (Lebre *et al*., 2005; Nagarajan *et al*., 2008; Nkwanyana *et al*., 2009) in an integrin-dependent manner (Hawkins *et al*., 2000; Cicala *et al*., 2009). The female genital tract anatomy will be discussed in more detail in Section 1.4.1

1.3 HIV mainly targets the mucosal immune system

A number of recent studies have highlighted the importance of the mucosa in HIV pathogenesis and HIV is now increasingly being recognized as a disease of the mucosal immune system (Musey *et al*., 2003a; 2003b; Derdeyn and Silvestri, 2005; Johnson and Kaur, 2005; Li *et al*., 2005; Mattapallil *et al*., 2005). While the vaginal and rectal mucosa are the predominant sites of HIV entry, the GALT is the site of initial HIV replication (Johnson and Kaur, 2005; Li *et al*., 2005; Mattapallil *et al*., 2005). During the early phase of simian immunodeficiency virus (SIV) and HIV infection, there is a rapid and widespread massive depletion of activated mucosal CD4+ T cells at mucosal sites and this occurs before significant depletion in blood and lymph nodes (Veazey *et al*., 2003). Recent evidence confirms that the level of CD4+ T cell depletion is far higher than at first anticipated, with 60-80% of memory CD4+ T cells depleted during early infection (Mattapallil *et al*., 2005). Thus, within the first few weeks of HIV infection, the virus targets the mucosal immune system and dramatically depletes the CD4+ T cells at this site. It has also been shown that HIV targeting of activated CD4+ T cells in mucosal tissues persists throughout infection, and not just in acute infection as previously thought. Mucosal tissues are likely to be a major source of viral replication, persistence and continual CD4+ T cell loss in HIV-infected individuals (Anton *et al*., 2003; Veazey *et al*., 2003; Picker *et al*.,

2004). It has also been shown that CD4+ T cells are not completely restored even after initiation of anti-retroviral therapy (Guadalupe *et al*., 2003; Brenchley *et al*. 2004; Mehandru *et al*., 2004), suggesting that antiretroviral drugs only partially control viral replication at mucosal sites. Reduced CD4+ T cell frequencies during chronic HIV infection have also been shown in other mucosal sites such as rectal mucosa (Critchfield *et al*., 2008), male genital tract (Politch *et al*., 2009), female genital tract (Veazey *et al*., 2003; Nkwanyana *et al*., 2009) and lung mucosa (Vajdy *et al*., 2001)

1.4 The female genital tract and HIV transmission

Although vaginal intercourse carries a lower HIV transmission probability per exposure event (1 in 200 to 1 in 2000) than anal intercourse or parenteral inoculation, it contributes more new HIV cases (Hladik and Hope, 2009). Knowledge of the anatomy of the female genital tract is relevant for the understanding of HIV transmission events at this site.

1.4.1. Anatomy of the female genital tact

The lower genital tract in females consists of four distinct regions: (i) the keratinised introitus vaginal opening, (ii) the vaginal mucosa, (iii) the ectocervix, and (iv) the endocervix (Figure 1.2; Pudney *et al*., 2005; Hladik and McElrath, 2008). The transformation zone represents an abrupt transition between the ectocervix and the endocervix and contains the largest number of lymphocytes in the female lower genital tract (Edwards and Morris, 1985; Hladik and McElrath, 2008). The vaginal mucosa and ectocervix are covered by a multilayered squamous epithelium, which may provide better mechanical protection against invading pathogen compared to the single-layer columnar epithelium of the endocervix if intact (Hladik and Hope, 2009). However, the surface area of the vaginal wall and ectocervix is approximately 15 times larger than that of the endocervix, providing larger epithelial area for pathogen invasion (Pendergrass *et al*., 2003). Compared with the vagina, the ectocervical mucosa contains higher numbers of CD4+ T cells and CD1a+ Dendritic cells (DCs; Pudney *et al*., 2005), making this site vulnerable to HIV transmission (Coombs *et al*.,

2003). High concentrations of CD8+ T cells, natural killer cells and antigen-presenting cells in the ectocervix and transformation zone suggest the possibility of these sites being the predominant locations for the induction of effector CTL responses in the lower genital tract (Pudney *et al*., 2005). Although the endocervix has a thin epithelial lining, it is lined with mucus and innate defences that can protect against invading pathogens (Shacklett *et al*., 2009).

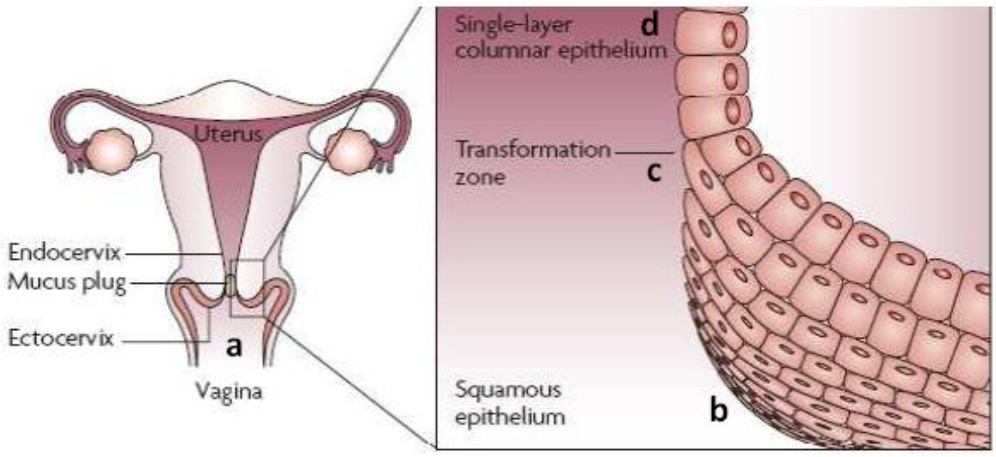

Figure 1.2 The architecture of the female genital tract. The vaginal mucosa (a) and ectocervix (b) are covered by a multilayered squamous epithelium. The transformation zone (c) represents an abrupt transition between the ectocervix and the endocervix and is highly enriched with lymphocytes. The endocervix (d) has a single-layer columnar epithelium lined with mucus (adapted from Hladik and McElrath, 2008).

1.4.2 HIV transmission across the female genital mucosa

The relative contributions of the vaginal, ectocervical, and endocervical mucosa to successful transmission remain unknown, but HIV penetration and infection have been demonstrated in all three sites (Hladik and Hope, 2009). An intact vaginal epithelium can serve as an efficient barrier to viral penetration (Shattock and Moore, 2003; Miller *et al*., 2005). In addition, cervical mucus has been shown to be very effective in trapping HIV-infected seminal cells or free virus (Maher *et al*., 2005; Miller *et al*., 2005). Despite the well-organized barrier at this mucosal site, HIV still gains entry to the female genital tract. Studies aimed at the identification of target cells mediating initial HIV entry across genital tissues have often come up with

conflicting results (Pope and Haase, 2003; Broliden *et al*., 2009). Nevertheless, it is generally accepted that HIV can cross the epithelial barrier where the mucosal barrier is most easily breached, by mechanisms such as the micro-trauma associated with sexual intercourse that provide immediate access to target cells in the submucosa (Haase, 2010). Risk factors associated with successful transmission of HIV infection include: (i) epithelial damage as a result of trauma-related abrasions or lesions caused by the presence of sexually-transmitted infections (Augenbraun and McCormack, 1994; Kreiss *et al*., 1994; Grosskurth *et al*., 1995; Bomsel, 1997; Plummer, 1998); (ii) viral load in plasma and transmitting partner's genital secretions (Lee *et al*., 1996; Quinn *et al*., 2000); (iii) the use of hormonal contraceptives or hormonal changes associated with menstruation which cause the thinning of the genital epithelium (Martin *et al*., 1998; Mestecky *et al*., 2009); and (iv) abundance of activated HIV target cells (CD4+ T cells and Langerhans cells expressing CCR5) recruited along an inflammatory gradient as a result of trauma or sexually transmitted infections (Patterson *et al*., 1998; Meng *et al*., 2002; Pilcher *et al*., 2004; Pudney *et al*., 2005; Margolis and Shattock, 2006; Weiler *et al*., 2008;).

The mechanisms by which HIV establishes infection *in vivo* are still elusive but two main hypotheses are presented (i) cell-free virion infection of cells such as CD4+ T cells, macrophages and DCs (through CD4+ T cells and CCR5/ CXCR4); and (ii) cell-to-cell transfer which is more rapid compared to cell free infection (Pope and Haase, 2003). The transfer of HIV can be either from T cell to another T cell, macrophage to T cell and DC to T cell (Pope and Haase, 2003; Groot *et al*, 2008). DCs express C-type lectin receptors such as dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) which are involved in HIV attachment and internalisation into endosomal compartments (Turville *et al*., 2001). Langerin cells can protect or enhance HIV acquisition depending on the concentration of HIV (Ganor *et al*., 2010). When cells are weakly infected with HIV, HIV is internalized and degraded by Langerhans cells. In contrast, cells highly infected with HIV attract Langerhans cells to the mucosal surface which capture HIV, forming conjugates with T cells allowing transfer to T cells (Ganor *et al*., 2010). Infected DCs migrate to local draining lymph nodes and transmit the virus to CD4+ T cells present in these lymph nodes and subsequently causing the spreading of HIV infection

throughout the body (Spira *et al*., 1996; Miller *et al*., 2005). Figure 1.3 summarizes local and systemic events that occur after HIV transmission at mucosal surfaces.

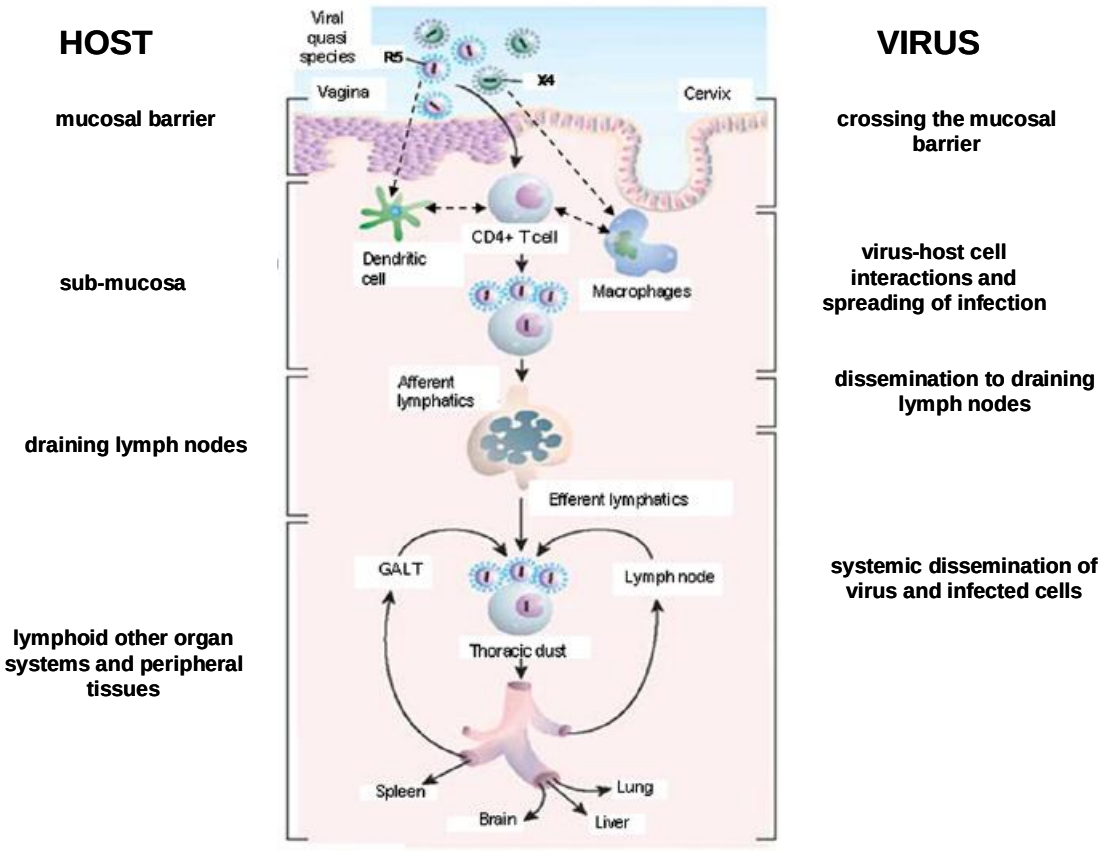

Figure 1.3 Local and systemic events that occur after HIV transmission at mucosal surfaces. After crossing the mucosal barrier, the first cells infected within the sub-mucosa include dendritic cells, CD4+ T cells and macrophages. Infected DCs migrate to draining lymph nodes where they infect lymphatic tissue reservoirs leading to systemic dissemination of the virus (adapted from Pope and Haase, 2003).

1.5 The importance of HIV-specific immune responses

Once HIV infection is established, HIV-specific cytotoxic T cells (CTL) are detected at several mucosal sites (Reynolds *et al*., 2005). Although the amount of HIV-specific CD8+ and CD4+ T cells show substantial inter-subject variation, several lines of evidence implicate systemic HIV-specific CD8+ T lymphocytes in the control of HIV infection (McMichael and Rowland-Jones 2001; Edwards *et al*., 2002). The HIV-specific CTL response has a significant impact on the outcome of HIV-1 infection, as suggested by: (i) dramatic rise in viral load following depletion of CD8+

T cells in SIV infected macaques (Jin *et al*., 1999; Schmitz *et al*., 1999), (ii) the strong association between specific human leukocyte antigen (HLA) types and viral load/disease progression (O'Brien and Nelson, 2004; Fellay *et al*., 2007), (iii) the decline in peak viraemia when virus-specific CD8+ T cells appear during primary infection (Borrow *et al*., 1994; Koup *et al*., 1994), and (iv) escape mutations that occur in CTL epitopes which are under positive selection (Price *et al*., 1997; Leslie *et al*., 2004) and lead to an increase in viral load (Oxenius *et al*., 2004).

Several studies have demonstrated the existence of mucosal CD8 T responses in the female genital tract and semen of HIV-infected individuals (Musey *et al*., 1997; Kaul *et al*., 2000; Shacklett *et al*., 2000a; 2000b; White *et al*., 2001; Kaul *et al*., 2003; Musey *et al*., 2003a) and some studies have suggested that these local CD8 responses may be important in local control of HIV infection. Cervical HIV-specific CD8 T cells at the genital mucosa have been shown to secrete cytokines [interferon (IFN)-γ] and are capable of cytolytic effector function in response to defined HIV epitopes in HIV-1 infected women (Musey *et al*., 1997; Kaul *et al*., 2000; Shacklett *et al*., 2000a; 2000b; Kaul *et al*., 2003; Musey *et al*., 2003a). Although there is no direct evidence of the control of HIV infection by CTLs in the genital tract, a study in HIV exposed but persistently sero-negative (HEPS) individuals has implicated HIV-reactive CTLs and genital HIV-specific neutralizing antibodies at the cervix in protection from HIV infection (Kaul *et al*., 2000). However, HIV-specific CTL in semen were associated with mucosal inflammation and increased HIV RNA shedding (Sheth *et al*., 2005).

Recently, it has emerged that T cells which secrete multiple cytokines and cytolytic mechanisms with high proliferative capacity are associated with better HIV disease outcome and it becoming clear that IFN-γ production alone by CD8 T cells may not be sufficient for HIV control (Harari *et al*, 2004; Pantaleo and Koup, 2004; Harari *et al*., 2006). The role played by genital tract anti-HIV CTLs in controlling HIV shedding in the female genital tract during HIV infection needs further investigation. Unfortunately, a study by Reynolds *et al*. (2005) in macaques acutely infected with SIV showed that, although HIV-specific CTLs become detectable within 14 days after HIV-infection in both the genital mucosa and in blood, these arise after the peak in plasma viremia has occurred and after a productive infection was established in blood.

In the absence of anti-retroviral treatment, the majority of HIV-infected individuals, (except for the few individuals termed progressive non-progressors or elite controllers) inevitably progress to AIDS even in the presence of anti-HIV CTLs. This suggests that although CTLs play an important role in the control of HIV, there are other factors associated with the virus itself that may drive HIV pathogenesis, leaving the host immune system overwhelmed. Understanding the mechanisms behind the failure of CTL based immune containment is therefore important.

1.6 Is there compartmentalization of HIV and CTL responses between the genital tract and blood?

Since the genital mucosa and blood represent distinct immunological environments, an important question is whether there is compartmentalization of HIV and HIV-specific CTLs in genital tract compartment compared to blood. A number of studies have reported distinct genetic populations of HIV in the genital tract compared to blood, suggesting some degree of compartmentalization exists (Poss *et al*., 1995; Overbaugh *et al*., 1996; Zhu *et al*., 1996; Coombs *et al*., 1998; Delwart *et al*., 1998; Kiessling *et al*., 1998; Poss *et al*., 1998; Gupta *et al*., 2000; Ping *et al*., 2000; Ellerbrock *et al*., 2001; Kovacs *et al*., 2001; Wright *et al*., 2001; De Pasquale *et al*., 2003; Kemal *et al*., 2003; Ghosn *et al*., 2004; Adal *et al*., 2005; Philpott *et al*., 2004; Sullivan *et al*., 2005; Andreoletti *et al*., 2007; Kemal *et al*., 2007; Diem *et al*., 2008). However, the fact that levels of HIV detectable in cervical secretions often correlates with viral load in blood suggests some mixing of genital and blood HIV-1 (Bull *et al*., 2009). The prevailing view today is that there are no strict physical barriers between the female genital tract and blood, with infected cells of specific phenotypes trafficking freely between compartments (Poss *et al*., 1998; Bull *et al*., 2009). This suggests that compartmentalization of HIV between blood and genital tract is only partial and that it may be due to proliferation of infected cells caused by local mucosal environment that favours HIV replication (Poss *et al*., 1998; Bull *et al*., 2009). Some of the local factors in the mucosal microenvironment that have been hypothesized to facilitate HIV replication in the genital tract independently from blood are: (i) the localized cytokine milieu, (ii) differing inflammatory signals; and (iii) the presence of

different immune cell types in these distinct compartments (Poss *et al*., 1998; Kovacs *et al*., 2001; Wright *et al*., 2001; Philpott *et al*., 2004).

HIV diversity observed between genital tract and blood compartments has raised questions of the extent to which mucosal and blood T-cell populations overlap in terms of specificity and clonality. Unfortunately, there are conflicting reports about the relationship between mucosal and blood antigen-specific CTLs with some studies finding differences in antigen-specificity between cervical and peripheral blood CTL (Shacklett *et al*., 2000b; Reynolds *et al*., 2005) while others have demonstrated that blood and mucosal CTL have largely overlapping HIV-1 epitope specificities (Kaul *et al*. 2000; Musey *et al*., 2003a; 2003b; Ibarrondo *et al*., 2005).

1.7 HIV pathogenesis

Studies of HIV/AIDS pathogenesis have long focused on the role of CD4+ T-cell depletion as the key marker of disease progression (Sodora and Silvestri, 2010). Earlier models predicted that CD4+ T cell depletion was directly caused by virus-mediated killing of infected cells, with the host immune system slowly failing to replenish the lost CD4 cells (Ho *et al*., 1995; Wei *et al*., 1995). However, in recent years, the role played by chronic immune activation and inflammation in HIV pathogenesis has become increasingly apparent, supporting a more complex pathogenic model (Appay and Sauce, 2008). The pathogenesis of HIV infection is now characterized by CD4+ T cell immunodeficiency in the context of generalized immune activation and dysregulation, with massive memory CD4 T cell infection and depletion during acute infection. This is followed by gradual loss of remaining CD4+ T cells caused by persistent immune hyper-activation. Activation of CD4 T cells results in increased target cells for the virus, excessive apoptosis of uninfected T cells, generalized immune dysfunction and paradoxically impaired ability to control HIV replication (Picker and Watkins, 2005; Appay and Sauce, 2008). In addition, one of the key characteristics of the immune system of HIV-infected individuals is the loss of regenerative capacity and an accumulation of ageing T cells (Marandin *et al*., 1996; Jenkins *et al*., 1998; Moses *et al*., 1998).

The high levels of immune activation consistently found during pathogenic HIV infection compared to significantly lower levels of activation found during non-pathogenic SIV infection in natural primate hosts and during less pathogenic HIV-2 infection in humans, supports the strong association between immune activation and HIV pathogenesis (Sousa *et al*., 2002; Chakrabarti, 2004; Rowland-Jones and Whittle, 2007; Pandrea *et al*., 2008). During SIV infection, rhesus macaques which are not the natural hosts to SIV, exhibit progressive CD4+ T cell depletion and progression to AIDS with high levels of T cell activation (Chakrabarti, 2004; Pandrea *et al*., 2008). In contrast, SIV-infected sooty mangabeys and African green monkeys which are the natural hosts of SIV, do not develop any immunodeficiency, exhibit minimal T cell activation despite evidence of high levels of viral replication (Chakrabarti, 2004; Pandrea *et al*., 2008). Experimentally induced immune activation in natural primate hosts of SIV resulted in significant increases in viral replication and CD4+ T Cell depletion (Pandrea *et al*., 2008). Furthermore, most HIV-2-infected individuals have low viral load and robust immune responses and they usually display significantly less immune activation compared to HIV-1-infected individuals (Sousa *et al*., 2002; Rowland-Jones and Whittle, 2007).

Immune activation in HIV infection is a rather broad expression that covers a large range of events or observations involved in active molecular and cellular processes (Appay and Sauce, 2008). Hyper-immune activation during chronic HIV infection is multi-causal and is still not completely understood. Previous studies have indicated that some of the causes may be (i) antigenic stimulation by the HIV virus itself for example, Envelope protein gp120, accessory protein Nef (Rieckmann *et al*., 1991; Lee *et al*., 2003), (ii) co-infection with non-HIV pathogens, such as HSV-2, CMV and EBV (Doisne *et al*., 2004) (iii) homeostatic response to CD4 depletion at mucosal sites (Jameson, 2002) (iv) microbial translocation from the gut to the systemic immune system (Brenchley *et al*., 2006). Translocation of bacterial products is highly likely to result in an excessive activation of the innate immune response and stimulation of macrophages and dendritic cells to produce a range of proinflammatory cytokines such as TNFα, IL-6 and IL-1β (Appay and Sauce, 2008).

Immune activation can be determined by either (i) cellular immune activation markers, (ii) proliferation markers, or (iii) soluble activation markers (Appay and

Sauce, 2008). However, these different markers of immune activation may represent distinct pathologic mechanisms. Soluble activation markers (inflammatory cytokines) relevant to this dissertation and their possible involvement in HIV pathogenesis will be discussed in section 1.8.

1.8 Determinants of HIV shedding in the female genital tract

A number of studies have shown that plasma viral load is one of the most important predictors of HIV shedding in the genital tract, even among HIV-infected women taking anti-retroviral therapy (Speck *et al*., 1999; Tachet *et al*., 1999; Quinn *et al*., 2000; Bourlet *et al*., 2001; Rebbapragada *et al*., 2007; Sheth *et al*., 2004; Sheth *et al*., 2005). Although HIV-1 RNA levels in blood and mucosal compartments were correlated overall, some participants clearly demonstrated disproportionate shedding of HIV-1 RNA in mucosal compartment (Sheth *et al*., 2005). Local factors in the genital tract may influence local viral replication. In addition to plasma viral load, genital tract inflammation and sexually transmitted infections are known to be important contributors to HIV shedding and transmission in the female genital tract (Spear *et al*., 2008).

1.8.1 Association between genital tract inflammation and HIV shedding

Immune activation, which usually reflects the mounting of an anti-viral immune response, may be seen as a positive response to infection. However, such responses may ironically also contribute to HIV pathogenesis by supplying HIV virus with activated target cells (Lawn *et al*., 2001). Previous *in vitro* studies showed that proinflammatory cytokines including IL-1β, TNF-α and IL-6 can induce increase replication of HIV by activating the nuclear factor kappa B (NF-κB) which is known to be an enhancer of HIV replication (Osborn *et al*., 1989). It has also been shown that the genital tract of chronically HIV-infected women have significantly higher concentrations of inflammatory mediators than uninfected women (McGowan *et al*., 2004; Nkwanyana *et al*., 2009) and this is likely to be associated with increased levels of mucosal CD4+ T cell activation and genital tract viral replication and shedding. Recently, Nkwanyana *et al*. (2009) showed that inflammation is associated with

recruitment of CD4+ T cells which may become susceptible targets for HIV infection. The association between high concentrations of mucosal pro-inflammatory cytokines and increased mucosal HIV shedding has previously been observed (Reka *et al*., 1994; Al-Harthi *et al*., 1997; McGowan *et al*, 2004; Rebbapragada *et al*., 2007). While these studies have suggested that local inflammation associated with concomitant genital tract infections and/or immune responses to these infections increase HIV shedding, the specific immune/inflammatory mediators that are associated with genital HIV shedding have not been well characterized. Furthermore, the causal relationship between inflammation and HIV shedding remains unclear, with some studies suggesting that inflammation results in local shedding of HIV, whilst others suggest that HIV increases concentrations of inflammatory cytokines in the genital tract (Rautonen *et al*., 1994; Osborn *et al*., 1989; Cheung *et al*., 2008; Spear *et al*., 2008).

1.8.1.1 IL-1β

IL-1β is predominantly produced by macrophages, monocytes, fibroblasts and dendritic cells (Dunn *et al*., 2001). IL-1β has been shown to be important for the inflammatory response of the body against infection by increasing the expression of adhesion factors on endothelial cells to enable transmigration of leukocytes (Dinarello, 1994; Copeland, 2006). IL-1β has also been shown to enhance HIV replication through direct activation of NF-κB (Osborn *et al*., 1989). IL-1β was shown to be associated with detection of proviral HIV and cell-associated HIV RNA in cervico-vaginal secretions from HIV-infected women (Zara *et al*., 2004). In addition, Poli *et al*. (1994) showed that IL-1β can synergize with IL-6 in enhancing HIV expression of latently infected human promonocytic cell line U1. On the other hand, HIV gp120 has also been suggested to induce production of IL-1β directly in a CCR5-dependent manner (Cheung *et al*., 2008).

1.8.1.2 IL-6

IL-6 is produced mainly by epithelial cells, macrophages, and T cells induced upon nuclear translocation and promoter binding of NF-κB (Van Snick, 1990). Nakajima *et al*. (1989) showed that IL-6 was produced primarily by monocytes during HIV infection. Nakajima *et al*. (1989) also showed that IL-6 has direct effects on immune

cells that include their activation and differentiation. IL-6 has also been shown to induce HIV replication by both transcriptional and post-transcriptional mechanisms and can also exacerbate inflammation by inducing co-expression of other proinflammatory cytokines (Poli *et al*., 1990; Spear *et al*., 2008). Conversely, HIV Tat has been shown to induce expression of various cytokines including IL-6 and TNF-α (Sastry *et al*., 1990; Rautonen *et al*., 1994).

1.8.1.3 IL-8

IL-8 is a proinflammatory CXC chemokine associated with the promotion of neutrophil chemotaxis and degranulation (Waugh and Wilson, 2008). Utgaard *et al*. (1998) reported that IL-8 was primarily produced by monocytes, although it was also found to be secreted by macrophages, endothelial and other cell types such as epithelial cells. Recently, Waugh and Wilson (2008) showed that expression of IL-8 was primarily regulated by NF-κB-mediated transcriptional activity. However, Hepatitis C virus and HIV envelope proteins were shown to collaboratively mediate IL-8 secretion in an NF-κB-independent manner, through activation of p38 mitogen activated protein (MAP) kinase and the tyrosine phosphatise (Balasubramanian *et al*., 2003).

1.8.1.4 IL-10

IL-10 is mainly produced by T cells, monocytes and B cells (Moore *et al*., 2001). In humans, studies have shown that IL-10 effectively down-regulates pro-inflammatory immune response resulting in its classification as a regulatory cytokine (Moore *et al*., 2001; Blackburn and Wherry, 2007). However, studies have also shown that IL-10 may have suppressive effects on the ability of cytotoxic T lymphocytes to kill the virus and this immunosuppression could lead to viral persistence *in vivo* (Olaitan *et al*., 1998, Brooks *et al*., 2006).

1.8.1.5 TNF-α

TNF-α is secreted by macrophages, monocytes, neutrophils, T-cells and NK-cells following their stimulation by bacterial lipopolysaccharides (Spurlock, 1997). TNF-α has been shown to modulate expression of IL-6 (Mire-Sluis, 1993). Mire-Sluis (1993) showed that expression of TNF-α was induced by the IFN-γ but inhibited by IL-6. TNF-α has been shown to increase HIV-1 proviral transcription (Crowley-Nowick *et*

al., 2000). In addition, elevated TNF-α may contribute to immunopathogenesis and up-regulate HIV viral replication through activation of NF-κB (Aukrust *et al*., 1994; Osborn *et al*., 1989). On the other hand, it has also been shown that HIV-1 gp120-induced TNF-α production by primary human macrophages through phosphatidylinositol-3 (PI-3) kinase and MAP kinase pathways (Lee *et al*., 2005).

1.8.2 Relationship between sexually transmitted infections and genital HIV shedding

A number of studies have found that vaginal infections with other sexually transmitted infections or bacterial vaginosis have a facilitating role in local HIV viral replication and shedding by interacting with the local mucosal immune environment (Donders *et al*., 2002; Zara *et al*., 2004; Mitchell *et al*., 2008). Donders *et al*. (2002) reported that *Candida albicans* induced TNF-α in the genital tract while aerobic bacterial infections were associated with elevated concentrations of IL-6 and IL-1β in vaginal fluids. Mitchell *et al* (2008) found that concomitant bacterial vaginosis and not HIV infection was directly responsible for elevated IL-1β levels in vaginal fluid from HIV-infected women. Other studies have confirmed that bacterial vaginosis is associated with the HIV shedding in the genital tract (Cu-Uvin *et al*., 2001) and bacterial vaginosis and *Candida* vaginitis increased the level of HIV being shed in the female genital tract in HIV-infected women (Sha *et al*., 2005). Bacterial vaginosis has been associated with high concentrations of IL-1β and TNF-α which has been suggested to be the mechanism by which this risk factor enhances HIV shedding (Sturm-Ramirez *et al*., 2000). Lipopolysaccharides released from these opportunistic pathogens at the cervical mucosa may result in the elevation of pro-inflammatory cytokines and subsequently the activation and proliferation of T cells (Tough *et al*., 1997). Bacterial vaginosis-associated disruptions in vaginal flora may also stimulate HIV replication through a heat-stable HIV inducing factor that increases replication by enhancing virus expression via activation of the HIV long terminal repeat (Cohn *et al*., 2005).

Herpes simplex virus type 2 (HSV-2) infection results in a persistent localized inflammatory response in the dermis below the healed lesion, consisting of HSV-2–specific CD4 T cells that express CCR5 or CXCR4, which are important HIV co-

receptors (Moriuchi *et al*., 2000; Zhu *et al*., 2009). Furthermore, HSV-2 suppressive therapy was shown to significantly reduced plasma viral loads and reduce genital tract HIV shedding in HIV-infected women co-infected with HSV-2 (Anderson and Cu-Uvin, 2008). Another recent study showed that men with larger lesions, purulent, multiple ulcers, higher plasma viral loads, and HSV-2 seropositivity were at increased odds of HIV-1 shedding after adjusting for other factors (Paz-Bailey *et al*., 2010). There was a significant correlation between HIV shedding and HSV-2 shedding in HIV infected women who were co-infected with HSV-2 (Rebbapragada *et al*., 2007). HSV2 viral DNA was also positively associated with increased chemokine levels [interferon inducible protein-10 (IP-10), macrophage chemotactic protein (MCP), regulated on activation normal T-cell expressed and secreted (RANTES) and monokine induced by interferon (IFN)-γ (MIG)] and the frequency of CCR5+ CD4+ cells (Rebbapragada *et al*., 2007). Rebbapragada *et al*. (2007) concluded that the increase in CCR5 cells and chemokines during episode of HSV-2 shedding may recruit activated CD4 T cells to the genital tract, and therefore increase local replication of HIV.

HPV infection of the cervix may influence HIV pathogenesis by inducing the production of immune and inflammatory factors that enhance HIV expression (Gage *et al*., 2000; Smith *et al*., 2010). A meta-analysis was recently performed on 39 different studies that reported the effect of genital tract infections on the detection of HIV shedding in the genital tract (Johnson and Lewis, 2008). In this meta-analysis, HIV-1 detection in the genital tract was increased most substantially by urethritis and cervicitis. Cervical discharge or mucopus, gonorrhoea, chlamydial infection and vulvovaginal candidiasis were also significantly associated with HIV shedding. Other infections and clinical conditions were found to have no significant effect on the detection of HIV, although HSV-2 shedding was found to increase the concentration of HIV shedding, and genital ulcer disease was found to increase the odds of HIV detection significantly (Johnson and Lewis, 2008). Overall, this analysis revealed that infections that are associated with significant increases in lymphocytes recruitment to the genital tract are also associated with significant increases in HIV shedding (Johnson and Lewis, 2008).

1.9 Memory T-Cell differentiation and functional heterogeneity

During the immune response to infection, there are alterations in the characteristics of anti-viral CTL, reflected in phenotypic as well as functional changes (Appay and Rowland-Jones, 2004). This means that memory T cell pool will vary in their quality of response depending on their phenotype and cytokines secreted. Previous studies have shown that the stages of HIV-specific CD8+ and CD4+ T cell differentiation may be an important qualitative assessment (Champagne *et al*., 2001; Addo *et al*., 2007; Northfield *et al*., 2007; Potter *et al*., 2007), suggesting a link between differentiation status of T cells and functionality. The term "differentiation" means changes in the expression of genes that control cell cycle, survival, migration and effector functions of cells (Lanzavecchia and Sallusto, 2002; Appay and Rowland-Jones, 2004). Elucidation of this process of T-cell differentiation and its association with control of viral infection is important for our understanding of T-cell immunity (Appay and Rowland-Jones, 2004).

1.9.1. Memory T subsets and their functional characteristics

A number of subsets of T cells have been described based on both their phenotypic and functional characteristics (Appay and Rowland-Jones, 2004). Naïve T cells (Tn) are thought to be long-lived cells that have never responded to their cognate antigen and are characterised by expression of CD45RA, the lymph node homing receptors CCR7 and CD62L (Appay and Rowland-Jones, 2004). Memory T cells respond more quickly to antigenic stimuli compared to Tn cells, and usually express CD45RO (Connors et al., 1997). Memory T cells can be further divided into central memory (TCM) and effector memory (TEM) T cells (Sallusto et al., 1999). TCM cells are long-lived cells that preferentially migrate to secondary lymphoid tissues due to their expression of CCR7 and CD62L, and have little or no immediate effector functions. Instead, they respond to antigen by rapidly dividing and differentiating into effector cells (Wherry *et al*., 2003; Kassiotis and Stockinger, 2004). Furthermore, TCM cells have self-renewal ability and are more likely to secrete interleukin (IL)-2 upon stimulation (Day and Walker, 2003; Lanzavecchia and Sallusto, 2005). Conversely, TEM cells are short-lived, can migrate to peripheral tissues because of their lack of CCR7 and CD62L expression, and have more rapid effector functions compared to

TCM cells, with the ability to secrete both IFN-γ and IL-2 (Day and Walker, 2003). Moreover, TEM cells have limited ability to proliferate and express pro-apoptotic genes (Masopust *et al*., 2001; Harari *et al*., 2004; Riou *et al*., 2007).

Another T cell subset that has been identified is terminally-differentiated effector (TEff) subset. These are T cells that have reached terminal stages of differentiation, re-express CD45RA and are more likely to die upon re-stimulation (Seder and Ahmed, 2003; Addo *et al*., 2007; Northfield *et al*., 2007, Riou *et al*., 2007). New T cell subpopulations continue to be detected as increasing numbers of cell surface markers (such as receptors involved in co-stimulation) are studied by multi-parameter flow cytometry (Appay and Rowland-Jones, 2004). Intermediate T cells (TInter) express CD45RA and CD27 but do not express CCR7 (Wills *et al*., 2002; Burgers *et al*., 2009). Transitional memory cells (TTM) express CD27 but do not express CD45RA and CCR7 (Verhoeven *et al*., 2008; Burgers *et al*., 2009). TTM cells are thought to be cells that are arrested between TCM and TEM phenotypes that may revert to a TCM phenotype or differentiate to a TEM phenotype and their direction of differentiation is dependent on the level of antigenic stimulation (Verhoeven *et al*., 2008). T cell differentiation and its association with HIV infection will be discussed in Section 1.9.3.

1.9.2 Models of memory T cell differentiation

Although different T subsets have been identified, there is little consensus on the pathways of memory/effector T-cell differentiation (Ahmed *et al*., 2009). Several models of T-cell differentiation have been proposed in recent years, as demonstrated in Fig. 1.4 (Champagne *et al*., 2001; Sallusto and Lanzavecchia, 2001; Kaech *et al*., 2002; Seder and Ahmed, 2003; Tussey *et al*., 2003; Wherry *et al*., 2003; Appay and Rowland-Jones, 2004; Lefrancois and Marzo, 2006; Ahmed *et al*., 2009). In the first model, a divergent pathway has been proposed whereby naive T cells give rise to daughter cells that develop into either effector or memory T cells. In this model, naive T cells can develop directly into memory T cells without becoming effector cells (Chang *et al*., 2007). In the second model, a linear-differentiation pathway has been proposed whereby memory T cells are direct descendants of effector cells. This

model suggests that memory T-cell development does not occur until antigen is removed (Kaech *et al*., 2002). In the third model, a short duration of antigenic stimulation favours the development of central memory T cells, whereas a longer duration of stimulation favours the differentiation of effector memory T cells (Catron *et al*., 2006; D'Souza and Hedrick, 2006; Badovinac and Harty, 2007). These models are contradictory and it has not yet been established which one is correct.

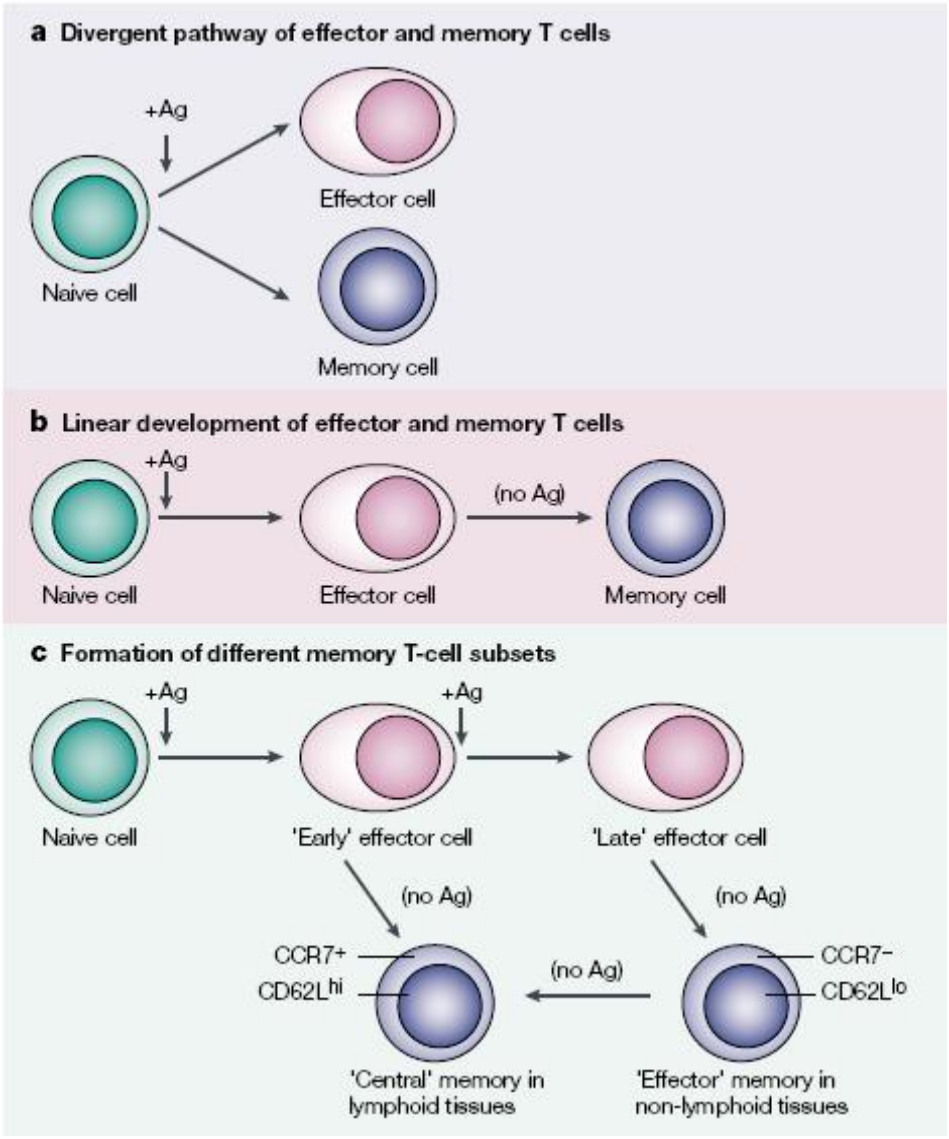

Figure 1.4 Models of memory T cell differentiation. A simplistic illustration of the currently debated models of T-cell differentiation is presented. (a) Model 1 represents divergent pathway whereby a naive T cell can give rise to daughter cells that develop into either effector or memory T cells. (b) Model 2 is a representation of the more conventional linear pathway of differentiation of naïve T cells into effector cells and ultimately memory cells. (c) Model 3 represents differentiation pathway that is dependent on the duration of antigenic stimulation whereby effector T cells can either develop into TCM cells when the duration of antigenic stimulation is short or differentiate into TEM cells when the duration of antigenic stimulation is long (taken from Kaech *et al*., 2002).

1.9.3. The impact of HIV infection on memory T cell heterogeneity

Previous studies have shown that not all CD4+T cells are equally targeted by HIV or support HIV replication, and this has been shown to depend on memory phenotype, activation status and pattern of expression of HIV co-receptors (Grossman *et al*., 2006; Picker, 2006; Dai *et al*., 2009;). Interestingly, T cell patterns of expression of HIV co-receptors was found to correlate with the cellular activation state and memory phenotype of the T cells, with the HIV co-receptor CCR5 being predominantly expressed by activated CD4+ TEM cells and CXCR4 being expressed mainly by resting Tn and TCM cells (Sallusto *et al*., 1998; Sallusto *et al*., 1999). This highlights the importance of the knowledge of phenotypes, activation status and functional characteristics of T cells in the female genital tract, a site that is critical for HIV pathogenesis and transmission. However, there are a very few studies that have described the memory differentiation status of T cells in the female genital tract and their relationship to HIV infection and replication (Saba *et al*., 2010).

A recent study by Saba *et al*. (2010) showed that the vast majority of T cells in human cervicovaginal tissue are of the CCR5 expressing TEM cells. Using an *ex vivo* model, Saba *et al*. (2010) showed that human cervicovaginal tissue preferentially supported the productive infection of activated CD4 T cells by R5-tropic HIV, which was followed by bystander activation of uninfected CD4 T cells that could subsequently become infected with HIV. These data highlight the dependence of HIV pathogenesis on the differentiation and activation state of cervicovaginal lymphocytes.

Chronic HIV-infection has been shown to be associated with gradual exhaustion of the T cell memory pool (Farrel, 2006; El-Far *et al*., 2008). Previous studies that were done in blood and mucosal tissues (such as lungs and GALT) have highlighted the importance of the preservation of long-lived TCM cells during chronic HIV and SIV infections (Potter *et al*., 2007; van Grevenynghe *et al*., 2008). In blood, HIV-infected individuals who exhibit control over plasma viraemia (termed HIV controllers) demonstrated a preserved TCM pool and a higher frequency of HIV-specific CD4+ TCM cells (Potter *et al*., 2007; van Grevenynghe *et al*., 2008). In contrast, HIV viremic individuals demonstrated a reduced CD4+ TCM pool (Ladell *et al*., 2008).

Some studies have suggested that in mucosal tissues, the maintenance of the TEM pool may depend on the supply of TCM cells for their replenishment (Marzo *et al*., 2007; Okoye *et al*., 2007). Verhoeven *et al*. (2008) showed that HIV-infected individuals with high frequencies of CD4+ TCM cells in GALT were best able to reconstitute their mucosal CD4+ T cell pools. However, it is not clear whether TCM cells proliferate at mucosal sites to produce TEM locally or whether TEM migrate to mucosal sites after differentiation, since TEM cells in blood have been shown to express high levels of integrin-α4β7 which engages mucosal vascular addressin cell molecule 1 (MAdCAM-1) on lamina propria endothelial cells (Hamann *et al*. 1994, Reiss *et al*. 2001). The role of integrin-α4β7 in the homing of effector T cells to the mucosal sites is supported by the observation that neutralizing antibodies to α4 and β7 integrin chains results in a reduction of lymphocytes in the intestine of mice (Hamann *et al*. 1994).

Increased numbers of highly differentiated T cells have been regarded as an indication of the onset of clinical immunodeficiency (Appay and Rowland-Jones, 2004). Papagno *et al*. (2004) showed that during chronic HIV-infection, individuals who had high frequencies of highly differentiated CD8+ T cells in blood also had reduced absolute CD4 T cell count. Over time, the accumulation of highly differentiated T cells due to persistent immune activation of memory T subsets may result in immune dysfunction, progressive decline of regenerative capacities and the development of immuno-senescence (Appay and Sauce, 2008).

While a large number of peripheral T cells end up dying upon activation, it should be noted that expansion and apoptosis in CD4+ and CD8+T cells may differ substantially (Homann *et al*., 2001; Foulds *et al*., 2002). Studies have shown that CD8+ T cells generally experience extensive expansion upon activation and can therefore establish a stable pool of resting memory cells (Homann *et al*., 2001) while the capacity of CD4+ T cells to expand and survive is lower resulting in the vast majority of activated CD4+ T cells dying rapidly (Homann *et al*., 2001; Foulds *et al*., 2002).

1.10 Mechanisms regulating memory T cell heterogeneity

The underlying mechanisms that cause the substantial degree of the heterogeneity in phenotype and function of memory T cells in general, remain unclear. A deeper understanding of the mechanisms responsible for the regulation of memory T cell phenotypes is essential for the generation of successful therapies during HIV infection aiming to strengthen the adaptive component of the immune system. Previous studies have suggested that inductive signals unique to distinct anatomical compartments may regulate memory T-cell differentiation in these compartments by providing a unique microenvironment that is the sum result of antigen persistence, the cytokine milieu, and the types of surrounding immune cells (Kundig *et al*., 1996; Iezzi *et al*., 1998; 1999; Jelley-Gibbs *et al*., 2000; Langenkamp *et al*., 2000; Kassiotis and Stockinger, 2004).

1.10.1 Antigen persistence

It has been proposed that the balance in the memory T cell population is directly associated with the level and duration of T cell receptor stimulation (Ahmed and Gray, 1996; Lanzarecchia and Sallusto, 2000; 2001; Kaech *et al*., 2002; Lanzarecchia and Sallusto, 2002; Gett *et al*., 2003; Lanzarecchia and Sallusto, 2005; Kalia *et al*., 2006), which in turn is likely influenced by the quantity of the antigen present. The result being that long-lived memory T cells are more preserved when the antigen load is effectively controlled. Based on these observations, the ‘decreasing-potential’ hypothesis has been proposed which states that the primary factor that distinguishes effector T cells that die from those that survive and differentiate into long-lived memory T cells is the duration and level of antigenic stimulation to which the T cells are exposed (Figure 1.5; Ahmed and Gray, 1996; Kaech *et al*., 2002).

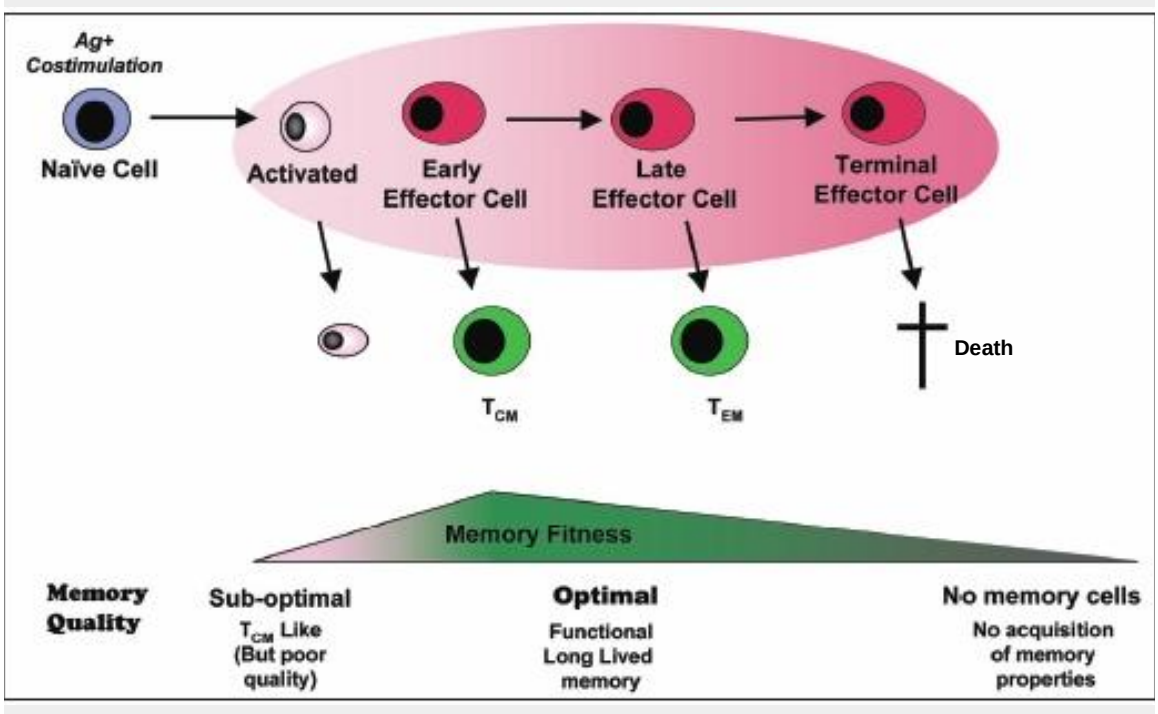

Figure 1.5 The decreasing-potential hypothesis. This model suggests that preservation of memory steadily decrease as a consequence of persisting antigen (as observed in chronic infections). Duration of antigenic stimulation increases from left to right. When antigenic stimulation is too short and antigen dose is too low, suboptimal memory is formed. Sufficient duration of antigenic stimulation favours the development of long-lived TCM memory whereas longer stimulation favours the differentiation of short-lived TEM cells. Terminally differentiated T cells are those that have received highest magnitude of antigenic stimulation and have the highest susceptibility to apoptosis with the lowest potential of differentiating into memory cells (Kaech *et al.*, 2002)

1.10.2 Inflammatory cytokines

In addition to the role played by antigenic stimulation, it has been shown that the type of cytokines present in the T cell microenvironment is an important factor in determining the extent of T cell differentiation (Sinigaglia and D′ambrosio, 2000). Pro-inflammmatory cytokines have been shown to drive differentiation from T helper (Th)0 cells to Th1 cells (Crowley-Nowick *et al.*, 2000). Furthermore, pro-inflammatory cytokines in the genital tract have been indirectly associated with recruitment, differentiation and activation of immune cells (Belec *et al.*, 1995; Sonza *et al.*, 1996; Crowley-Nowick *et al*, 2000; Nkwanyana *et al.*, 2009). *In vitro* studies have demonstrated that T cells are capable of proliferating in an antigen-independent manner in the presence of inflammatory cytokines (Unutmaz *et al.*, 1994; 1995).

Similarly, Geginat *et al*. (2001) showed that that *in vitro* addition of cytokines (such as TNF-α, IL-6, IL-10 and IL-12) resulted in TCM cells differentiating into TEM-like cells expressing CCR5. The cervical mucosal environment has high levels of inflammation, particularly in the presence of HIV-infection, as indicated by increased concentrations of inflammatory cytokines such as IL-6, IL-1β and IL8 in the genital tract compared to matching blood samples (McGowan *et al*., 2004; Nkwanyana *et al*., 2009). A question that arises from these findings is whether the high levels of inflammation in the female genital tract of HIV-infected women impacts on the distribution of memory T cells in HIV-infected compared to uninfected women.

1.10.3 Homeostatic cytokines

There is growing evidence that the common γ-chain (γc) cytokines (such as IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21) are involved in T cell maintenance and homeostasis (Caccamo *et al*., 2005; Gougeon and Chiodi, 2010). Homeostatic proliferation is a feature of both naive and memory T cells that takes place after significant loss of T cells (such as in the case of lymphopenia). This thesis will focus on the role of IL-15 and IL-7 in the regulation of T cells in the female genital tract.

1.10.3.1 IL-15

IL-15, which is produced by non-lymphoid cell types including DCs, is known to direct CD8+ memory T cell function and homeostasis (Kovanen and Leonard, 2004; Ahmad *et al*., 2005; Surh and Sprent, 2005). Previous studies have clearly implicated IL-15 in the regulation of both homeostatic and HIV-associated CD8+ memory T cell proliferation (Mueller *et al*., 2003). A study by Picker *et al*. (2006) showed that IL-15 induced proliferation of rhesus macaque CD4+ and CD8+ TEM cells with little effect on the naive or TCM cell subsets. In addition, the increased proliferation induced by IL-15 resulted in emigration of these cells into extralymphoid effector sites (Picker *et al*., 2006). This suggests that IL-15 preferentially expands those populations of T cells capable of localizing in extralymphoid effector sites and directly responding to pathogen invasion. Since CD4 TEM cells are severely depleted in extralymphoid effector sites during HIV and SIV infections, even in the presence of anti-retroviral drugs, the therapeutic use of IL-15 in conjunction with anti-retroviral treatment has been suggested for the reconstitution of mucosal CD4 (Picker *et al*., 2006).

1.10.3.2 IL-7

Several immune cell types produce IL-7 such as stromal and endothelial cells of the bone marrow and thymus (Kroncke *et al*., 1996), intestinal epithelial cells (Watanabe *et al*., 1995), follicular dendritic cells (Sorg *et al*., 1998), and keratinocytes (Heufler *et al*., 1993). Factors regulating the production of IL-7 from these cells, however, remain unclear. IL-7 has been shown to have a number of effects on both CD8+ and CD4+ T cells from peripheral tissues and thymus (Alderson *et al*., 1991), such as thymopoesis, proliferation, maturation, differentiation, memory genesis and long-term survival (Fry and Mackall, 2005; Guimond *et al*., 2005; Sieg *et al*., 2005). The essential role of IL-7 in the maintenance of naive T cell numbers both *in vitro* and *in vivo* and in the promotion of homeostatic division under conditions of lymphopenia, such as in HIV infection was initially described by Bradley *et al*. (2005). The role of IL-7 in T cell survival has also been demonstrated by its ability to up-regulate anti-apoptotic factors (such as Bcl-2 and Bcl-xl) as well as factors leading to cellular proliferation (Maraskovsky *et al*., 1997; Von Freeden-Jeffry *et al*., 1997; Fry and Mackall, 2005).

During chronic HIV-infection, however, high levels of IL-7 have been associated with CD4+ T cell depletion (Llano *et al*., 2001; Napolitano *et al*., 2001; Albuquerque *et al*., 2007). It is still not clear why IL-7 is not able to rescue the declining T cell pool during chronic HIV-infection. It has been suggested that the receptor for IL-7 (CD127) is down-regulated during chronic HIV infection due to cellular activation, causing memory T cells to be less responsive to IL-7 homeostatic signals (Wherry *et al*., 2004; Kiazyk and Fowke, 2008). Alternatively, others have argued that CD4+ T cell depletion drives the elevated production of IL-7 by immune cells (Fry and Mackall, 2005).

Because of its potent effect on survival and expansion of T cell populations, IL-7 is being developed as a therapeutic agent for T cell reconstitution (Nunnari and Pomerantz; 2005; Snyder *et al*., 2006; Sportes *et al*., 2008). Initial trials conducted in humans have suggested that therapy with recombinant human IL-7 (rhIL-7) may improve T cell function during HIV-infection (Sportes *et al*., 2008). There are some data from previous studies, however, on the biological effects of IL-7 that should be considered in the context of rhIL-7 therapy in HIV-infected individuals (Gougeon and

Chiod, 2010). Persistence of HIV reservoir cells was found to be driven by IL-7-mediated homeostatic proliferation (Chomont *et al*., 2009). Previous *in vitro* studies have shown that IL-7 may promote HIV replication in naturally HIV-infected peripheral blood (Smithgall *et al*., 1996). IL-7 has been shown *in vitro* to increase Fas (death receptor, also known as CD95) expression on naïve and memory T cells, followed by apoptosis upon Fas triggering (Fluur *et al*., 2007). It was also found that IL-7 is a potent inducer of IL6, IL-1α, IL-1β, and TNF-α by monocytes, suggesting that IL-7 may be an important mediator in inflammation (Alderson *et al*., 1991). The induction of inflammation by IL-7 was shown to be dependent on the concentration of IL-7 present. Low concentrations of IL-7 stimulated T cell growth and differentiation whereas at high concentrations of IL7 induced inflammatory cytokine secretion by monocytes (Alderson *et al*., 1991). Indeed, it remains to be determined whether these findings from *in vitro* experiments are representative of what may occur *in vivo* during therapeutic treatment with rhIL-7. On the other hand, the ability of T cells to respond to IL-7 depends on the expression of CD127. Since there are high levels of immune activation and reduction of CD127 expression by T cells during chronic HIV infection, administration of IL-7 might not be as beneficial with regard to the homeostatic expansion of reconstituting T cells (Kiazyk and Fowke, 2008). Furthermore, IL-7 itself leads to transient down-regulation of CD127 (Kiazyk and Fowke, 2008). This suggests that there is still a need for a clearer understanding about the associations between T cell subsets, survival receptor expression by T cells, homeostatic and inflammatory cytokines to determine whether rhIL-7 therapy would be feasible. More studies are warranted on how to maintain memory at mucosal sites where the levels inflammation are high and where homeostatic regulation of T cells may be more frequent and necessary during HIV infection due to high levels of CD4+ T cell depletion. Studies of the potential mechanisms that regulate T cells at the genital mucosa are important since there is limited information about what induces different T cell phenotypes in different microenvironments.

1.11 Study aims and objectives

The overall aim of this dissertation was to investigate the impact of inflammation on shedding and T cell quality (function, maturational status and CD4+ T cell decline) in the female genital tract during HIV infection.

Rationale for this study

The majority of new HIV infections are in women as a result of HIV invasion of the female genital tract via exposure to virus-containing semen (Hladik and Hope, 2009). In HIV-infected women, the female genital tract is a site from which HIV is transmitted to new sex partners. Mucosal tissues are also the major sites of CD4 depletion during HIV infection (Mattapallil *et al*., 2005). Although it is now becoming more widely accepted that the HIV virus has a more substantial impact on the mucosal immune system than its systemic counterpart, we still do not fully understand the mechanisms of HIV pathogenesis particularly at mucosal sites. The vast majority of work to understand HIV pathogenesis has relied on blood samples because they are easily accessible. However, blood compartment may not adequately reveal events occurring at mucosal sites. The studies in this thesis will focus on maturational status and functionality of T cells and the genital innate microenvironment (inflammatory and homeostatic cytokine profiles) within the context of HIV pathogenesis in the female genital tract during HIV infection. A deeper understanding of the mechanisms responsible for shedding and immune function defects of T cells is essential for the generation of successful therapies aiming to strengthen the adaptive component of the immune system and to prevent HIV transmission.

Specific objective 1

To investigate the association of HIV-specific CD8 T cells and inflammation with local genital HIV shedding in the female genital tract in HIV-infected women (Chapter 2). *Hypothesis*: HIV-specific CD8+ T cells in the female genital tract are associated with control of local HIV replication and shedding. Genital inflammation is associated with CD8+ T cell recruitment.

Specific objective 2

To investigate the impact of HIV infection and clinical status on the maturation (Chapter 3) and functional ability (Chapter 4) of T cells derived from female genital tract and blood. *Hypothesis*: Better control of HIV disease (less HIV shedding and high CD4 counts) results in less differentiation of T cells. Women who are controlling HIV have genital tract T cells with superior function (produce both IFN-γ and IL-2) and quality (long-lived memory T cells) than women who are not controlling HIV.

Specific objective 3

To investigate the role of inflammatory and homeostatic cytokines present in the female genital tract on the maintenance or reconstitution of mucosal CD4 T cell numbers and the differentiation status of cervical T cells (Chapter 5). *Hypothesis*: Local factors in the cervical microenvironment such as inflammatory cytokines and common cytokine receptor γ-chain family cytokines may have an impact on T cell differentiation profiles and CD4 T cell depletion in the genital mucosa.

Chapter 2

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Chapter 2
Impact of mucosal inflammation on cervical HIV-1-specific CD8 T cell responses in the female genital tract during chronic HIV infection

2.1 Summary

The female genital tract is the major route of heterosexual HIV acquisition and transmission. Here it was investigated whether HIV-specific CD8 T cell-mediated immune responses could be detected in the genital mucosa of chronically HIV-infected women; and whether these were associated with either local mucosal HIV shedding or local immune factors. It was found that CD8+ T cell IFN-γ responses to Gag were detectable at the cervix of HIV-infected women but that the magnitude of genital responses did not correlate with those similarly detected in blood. This indicates that *ex vivo* HIV responses in one compartment may not be predictive of those in the other. Increased genital TNF-α, IL-1β and IL-6 levels correlated significantly with levels of Gag-specific CD8+ T cells at the cervix. Women who were detectably shedding virus in the genital tract had significantly increased cervical levels of TNF-α, IL-1β, IL-6 and IL-8 compared to women who were not detectably shedding virus. However, there was no association between the magnitude of cervical HIV-specific responses and mucosal HIV shedding. These results support the hypothesis that pro-inflammatory cytokines in the female genital tract may promote HIV replication and shedding. In addition, it was further shown that inflammatory cytokines are associated with increased levels of HIV-specific CD8 effector cells at the genital mucosa but that these were not able to control genital HIV shedding.

2.2 Introduction

The female genital tract plays an important role in HIV-1 acquisition and transmission during heterosexual contact. Women appear to be at higher risk for infection than men, with an estimated 30% to 40% of annual worldwide infections occurring through HIV invasion of the female genital tract via exposure to virus-containing semen (Hladik and Hope 2009), with transmission risks for both sexes increasing with higher plasma viral loads (Fiore *et al.*, 2003). While plasma concentrations of HIV are a strong predictor of transmission rates, local mucosal factors such as genital inflammation and concurrent sexually transmitted infections (STIs) are also strongly associated with both increased shedding of virus at the genital tract and greater susceptibility to infection via the genital mucosa (Sha *et al.*, 2005; Cummins *et al.*, 2006; Coleman *et al.*, 2007; LeGoff *et al.*, 2007). Although immunological activation in response to invading pathogens is a crucial component of protective immunity, such responses may ironically also contribute to HIV pathogenesis by providing the virus with a steady supply of susceptible target cells (Lawn *et al.*, 2001).

Several lines of evidence implicate systemic HIV-specific CD8+ T lymphocytes in the control of HIV infection (McMichael and Rowland-Jones 2001; Edwards *et al.*, 2002). However, in some cases these HIV-specific CD8+ T cells fail to contain the virus and the majority of HIV-infected individuals, eventually progress to AIDS in the absence of anti-retroviral treatment. Mucosal HIV-specific CD8+ T cells have been detected both in semen and genital tracts of HIV-infected men and women, respectively. These cells are capable of cytokine production and cytotoxicity (Musey *et al.*, 1997; Quayle *et al.*, 1998; Kaul *et al.*, 2000; Shacklett *et al.*, 2000a; 2000b; White *et al.*, 2001; Kaul *et al.*, 2003; Musey *et al.*, 2003a; 2003b; Ibarrondo *et al.*, 2005; Sheth *et al.*, 2005; 2006; Critchfield *et al.*, 2007). Although no direct evidence exists for their role in local control of HIV infection, the presence of HIV-specific CD8+ T cells at the genital mucosa of exposed but uninfected commercial sex workers have implicated these cells in protection from HIV infection (Kaul *et al.*, 2000).

In this chapter, the interplay between mucosal HIV-specific CD8 T cells, local HIV shedding in the female genital tract and cervical inflammation was investigated. I compare the presence and magnitude of HIV-specific CD8+ T cells at the cervix with those in blood. Mucosal and systemic levels of TNF-α, IL-10, IL-1β, IL-6, IL-8 and IL-12p70 were measured and correlated to levels of HIV shedding in the genital tract. While HIV-specific CD8 T cells are detectable at the cervix of chronically HIV-infected women, this chapter shows that they probably do little to prevent HIV transmission. Instead, evidence supporting the role for cervical inflammation as the central effector of both CD8+ T cell recruitment and HIV shedding at the cervical mucosa is presented.

2.3 Materials and methods

2.3.1 Description of study participants

Fifty-one chronically HIV-1 infected women and 24 HIV negative women attending community clinics in Cape Town, South Africa were enrolled in this study. Women who were menstruating at the time of sampling, who were post-menopausal or had undergone a hysterectomy were excluded from the study. In line with South African National policy, syndromic management of sexually transmitted infections was followed in this study which does not take into account asymptomatic infections. This was done by excluding women who had vaginal discharge, visible ulcers or genital warts. The study was approved by the Research Ethics Committee of the University of Cape Town, South Africa (UCT REC REF 106/2002 and 258/2006) and informed written consent was obtained from all volunteers of the study. All participants were naïve to antiretroviral therapy.

2.3.2 Cervical mucosal sample collection and processing

Cervical mucosal mononuclear cells (MMCs) were collected using a Digene cervical sampler as described previously (Passmore *et al.*, 2002). Briefly, a Digene cervical cytobrush was inserted into the endocervical os (preferentially sampling from the transformation zone), rotated through 360° and immediately placed in 3 ml of cold

transport medium [RPMI1640 medium supplemented with 5mM glutamine, fungazone, penicillin, streptomycin and 10 % fetal calf serum (FCS)]. The cervical cytobrushes were transferred to 4oC in a Nalgene (Rochester, NY, USA) bench-top cooler until transported to the laboratory. Cervical samples that had visible red blood cell contamination were discarded. Cervical cells were isolated within four hours of collection by gently rotating the cytobrush against the sides of the tube to dislodge cells. Transport media was then flushed through the cytobrush bristles 30 times using a sterile plastic Pasteur pipette to dislodge all cervical-derived cells. The cell suspension was transferred to a sterile 15 ml centrifuge tube and the cells were pelleted at 1200 rpm (280 x g) for 10 min using a Heraeus Megafuge 1.0 R centrifuge. The supernatant fraction was stored at -80ºC until analysis for inflammatory cytokines and HIV shedding. Pelleted cells were re-suspended in 1.2 ml 10% FCS RPMI (transport medium). Cervical MMCs were counted by either (i) manual Trypan staining on a Naubauer Haemocytometer and light microscopy or (ii) automated Guava cell counter [200 µl of cells (16% of sample); Guava Technologies, Hayward, CA].

2.3.3 Manual counting using Trypan staining

Fifteen microlitres of Trypan blue dye (Sigma-Aldrich, Irvine, UK) was pipetted into a 96-well microtitre plates (Greiner Bio-one; Frickenhausen, Germany). Cervical cells were gently resuspended and an equal volume (15µl) was mixed with the trypan blue dye. Trypan blue/cell suspension (15µl) was loaded into a plastic counting chamber (Sigma-Aldrich, Irvine, UK). Using a Naubauer Haemocytometer and light microscopy, cervical mononuclear cells were counted under the 40× magnification from two opposite 4×4 squares. Non-viable cervical cells, with permeable cell membranes and took up the stain, appeared blue. Total number of cells was determined using the following equation:

$$\text{no. of cells/ml} = \frac{\text{Total no. of cells in two (4×4) squares}}{\text{no. of squares counted (2)}} \times \text{dilution factor(2)} \times 10^4$$

2.3.4 Automated counting of cervical cells using a Guava cell counter

Automated cell counting using a Guava cell counter to quantify fluorescently labelled CD3+ T cells is a useful and objective method to accurately quantify specific immune subsets within a mixed cervical cytobrush cell population (Nkwanyana *et al*., 2009). This method calculates absolute CD3+ T cell counts from a cytobrush sample with similar accuracy to the Becton-Dickenson Trucount flow cytometric method of determining absolute counts (Nkwanyana *et al*., 2009). Briefly, cells (25µl/tube) were stained with 2µl anti-CD3 PE monoclonal antibody (Guava technologies, Hayward, CA) and incubated at 4ºC for 30 minutes. Cells were washed with 1ml 1% FCS PBS and centrifuged at 1500 rpm (437 x g) for 5 minutes. The supernatant was discarded and 200ul CellFix (BD Biosciences) was added. At least 2000 events were acquired using an automated Guava cell counter and analysed using Cytosoft® software (Guava technologies, Hayward, CA). Cervical mononuclear cells were adjusted to ~1 x 10^6 cells per ml and rested for 16 hours at 37^{o}C and 5% CO_2.

2.3.5 Peripheral blood mononuclear cells (PBMCs) isolation

Blood samples from HIV-infected and uninfected women were collected using standard venipunture into sterile ACD anti-coagulated vacutainer tubes (BD Biosciences, Plymouth, UK)). PBMCs were isolated using Ficoll-Hypaque (Sigma-Aldrich, Egham, Runnymede, UK) density gradient centrifugation using LeucoSep® centrifuge tubes (Greiner Bio-one, Frickenhausen, Germany). Ficoll-Hypaque (15 ml), pre-warmed to room temperature, was added into a 50ml Leucosep tube with a filter disc and centrifuged at 2500rpm (1215 x g) in a Heraeus Megafuge 1.0 R centrifuge for one minute to allow the Ficoll to move below the disc. To each Leucosep tube (containing Ficoll), 30 ml whole blood was gently overlaid onto the disc. The tubes were centrifuged at 2300rpm (1029 x g) for 15 minutes. Using a disposable sterile plastic Pasteur pipette, plasma was carefully removed to avoid disturbing the PBMC 'buffy' layer. Plasma was aliquoted into cryovials and stored at -80ºC for later measurement of plasma HIV RNA levels. The “buffy” layer above the immobilized Leucosep disc enriched for PBMCs was removed using a Pasteur pipette and transferred into a 50ml falcon tube. PBMCs were washed twice in 25ml phosphate buffered saline (PBS), by centrifuging at 1200 rpm (280 x g) for ten

minutes. After the final wash, PBMCs were resuspended in 10ml RPMI1640 medium supplemented with 5mM glutamine, fungazone, penicillin, streptomycin and 10 % FCS. Cells were counted using an automated Guava cell counter.

2.3.6 Counting of PBMCs using an automated Guava cell counter

PBMCs (10µl) were added to 190µl Guava Viacount reagent (Guava technologies, Hayward, CA) and vortexed to mix thoroughly. Cells were incubated at room temperature for 8 minutes. Cells were acquired using an automated Guava cell counter and analyzed using Cytosoft® software (Guava technologies, Hayward, CA). The viable cell concentration was adjusted to 2 x 10^6 cells/ml and rested for 16 hours at 37^{o}C 5% CO_2. All experiments were carried out on fresh PBMCs.

2.3.7 Stimulation with HIV-1 Gag peptides and intracellular cytokine staining

PBMCs (2.0×10^6 cell/ml) or cervical cells (~$0.1\text{-}1.0\times10^6$ lymphocytes/ml) were stimulated with either (i) a single pool of 121 HIV subtype C (Du422) Gag overlapping peptides (used at a final concentration of 1 µg/ml (Currier *et al*., 2008); peptides were kindly provided by the NIH AIDS Reagent Repository); (ii) PMA/ionomycin (10 µg/ml; Sigma-Aldrich; positive control); or (iii) untreated for 6 hours at 37^{o}C and 5%CO_2. Brefaldin A (10 µg/ml; Sigma, St. Louis, MO) was added after the first hour. Following stimulation, the cells were washed once by adding 2 ml of 10% FCS PBS (0.01% NaN_3) by centrifuging for 5 min at 300×g (1300 rpm) at room temperature. Pelleted cells were resuspended in the dead volume and stained with phenotypic markers APC-labelled anti-CD3 (Becton-Dickinson, San Jose, CA) and FITC-labelled anti-CD8 (BD) for 30 minutes on ice. Cells were washed once by adding 2 ml of 10% FCS PBS (0.01% NaN_3) and centrifuged for 5 min at 300×g (1300 rpm) at room temperature. Surface stained cells were then fixed and permeabilized using BD CytoFix/CytoPerm for 10 minutes at room temperature and washed once with 0.1% Saponin (Fluka) PBS (containing 5% FCS and 0.01% NaN_3). Cells were stained with PE-conjugated anti-IFN-γ (BD) for 30 minutes on ice. Cells were finally washed with 2 ml of 10% FCS PBS (0.01% NaN_3), centrifuged (5 minutes, 300×g, 1300 rpm, room temperature) and fixed with BD Cell Fix. Cell fluorescence was measured using a FACSCalibur flow cytometer (BD

Immunocytometry Systems) and FlowJo software version 8.5.3 (Tree Star, Inc; Ashland, Oregon, OR, USA) was used for colour compensation and data analysis.

2.3.8 Measurement of inflammatory cytokine concentrations

Concentration of TNF-α, IL-10, IL-1β, IL-6, IL-8 and IL-12p70 in matched cervical and plasma samples from the 51 chronically HIV-infected women included in this Chapter were determined using a Human Inflammation Cytometric Bead Array (CBA) kit (BDIS) according to manufacturer's instructions. The limit of detection of this assay ranged between 1.9pg/ml and 7.2pg/ml (average 3.6pg/ml). Cytokine values below the assay's limit of detection were reported as 1.6 pg/ml (the mid-point between the average lower level of detection 3.6pg/ml and 0).

2.3.9 Determination of viral load in cervical supernatant and plasma

Viral loads in cervical samples were measured by evaluating HIV RNA copies in the supernatant of 3ml R10 tissue transport medium in which cells and mucous was flushed off the brushes and centrifuged [1200 rpm (280 x g) for 10 min]. Using cytobrushing for the collection of cervical cells and mucous in this study had an advantage of simultaneously assessing cervical immune cell function, inflammatory cytokine and HIV RNA concentrations from the same location within the female genital tract. However, the limitation to this method is that these viral loads were not normalized to the original volume of cervical mucous obtained by cytobrushing. HIV load in cervical supernatants and plasma samples was determined using Nuclisens Easyq HIV 1 Version 1.2 by the NHLS Diagnostic Virology Laboratory (Groote Schuur Hospital, Cape Town, South Africa). The detection limit of this assay is 50 HIV RNA copies/ml.

2.3.10 Statistical analysis

Mann–Whitney U test was used for comparison of unpaired non-parametric data. Paired T test was used for comparison of matched data. The Students' unpaired t test was used for comparison of unpaired parametric data. Spearman Rank test was used to test for correlations. All statistical tests were performed using GraphPad Prism 5®

(GraphPad Software, San Diego California USA). All tests were two-tailed and P-values ≤ 0.05 were considered significant.

2.4 Results

Fifthy-one HIV-infected and 24 uninfected women were included in this study. The median age of HIV-infected women was 31 years (IQR 28-37) versus 34 years for uninfected women (IQR 28-44) p=0.26. The 51 HIV-1 infected women included in this Chapter (Table 2.1) were chronically HIV-1 infected and had been sero-positive for more than three years. None were on anti-retroviral therapy at the time of study. They had a median CD4 count of 391 (IQR 319-528) cells/μl and a median HIV plasma viral load of 3400 (IQR 0-21000) RNA copies/ml, with 35.3% of these women having undetectable plasma viral loads despite being therapy naive, suggesting that these women may possibly be HIV controllers.

Table 2.1 Clinical details of chronically HIV-1 infected women included in the study

Characteristics	
N	51
Age in years (mean ± SD)	32.8 ± 6.2
Absolute blood CD4 count (cells/ml; median, IQR)	391 (319-528)
Plasma viral load (RNA copies/ml; median, IQR)	3400 (0-21000)
Number of women with detectable plasma viral load (N; %)	33/51 (64.7%)
Range of plasma viral load in women with detectable levels (RNA copies/ml)	80 – 72000
Number of women with detectable cervical viral load (N; %)	20/44[a] (45.5%)
Cervical viral load only in women with detectable levels (RNA copies/ml)	770 (270-1600)
Range of cervical viral load in women with detectable levels (RNA copies/ml)	92 – 44000

[a]Cervical supernantant samples were only available for viral load assessment from 44/51 of the HIV-infected women.

2.4.1 HIV-1 shedding in the female genital tract during chronic HIV-infection

Of the 51 women included 33/51 (64.7%) had detectable HIV RNA in their plasma samples had viral loads that ranged from 80 – 72000 RNA copies/ml (Table 2.1). Viral loads were determined from cervical supernatants of 44/51 of these women. Of these, 20/44 women (45.5%) had detectable HIV RNA in their cervical supernatant samples and were therefore classified as shedding virus. In these twenty women, the median HIV cervical viral load was 770 RNA copies/ml (ranging from 92 – 44000 copies/ml). There was a significant positive correlation between plasma and cervical viral loads ($p<0.0001$; Figure 2.1A) and HIV-infected women who were shedding HIV at the cervical mucosa had significantly higher plasma viral loads than women who were not shedding virus at the cervix ($p=0.0009$; Figure 2.1B). When women with an undetectable plasma viral load were excluded from the analysis, there was still a significant positive correlation between cervical viral load and plasma viral load ($p=0.03$, $Rho=0.4$; data not shown).

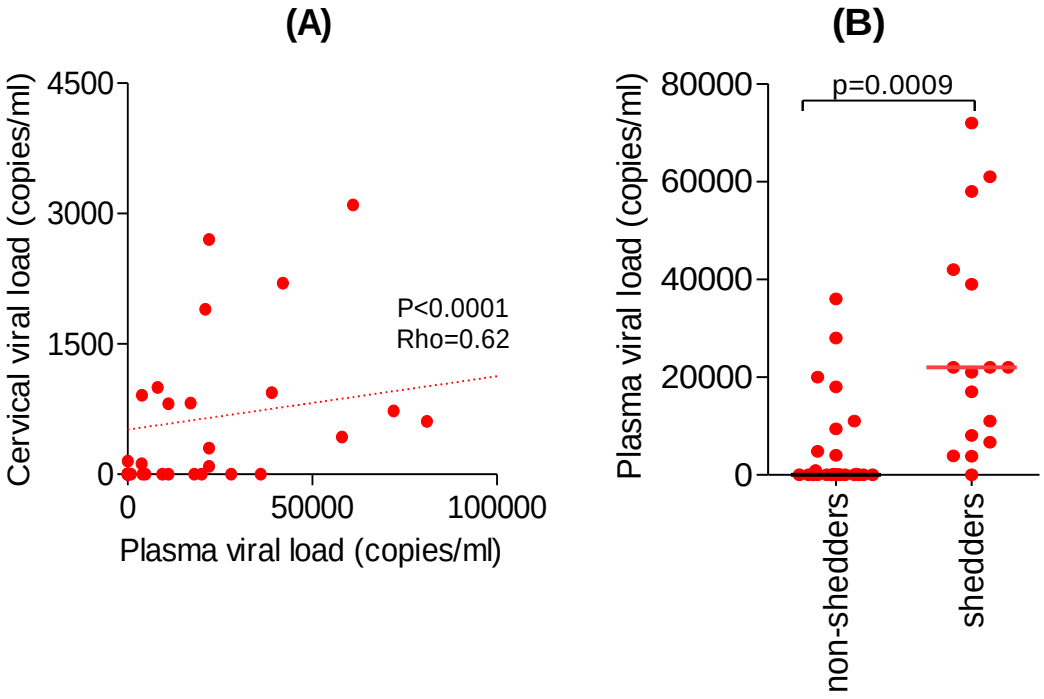


Figure 2.1. Correlation between plasma viral load and HIV shedding at the cervical mucosa during chronic HIV infection. (A) Association between cervical and plasma HIV loads. (B) Comparison of plasma viral load in women who had detectable HIV in their cervical supernatants (shedders) versus those that did not (non-shedders). Each data point represents an individual's plasma viral load. The line indicates the median viral load for each group. Spearman Rank test was used to test association between viral loads at cervix and plasma. Mann-Whitney U test was used to compare the plasma viral load between shedders and non-shedders.

2.4.2 Magnitude of HIV-1 Gag-specific CD8 T cell responses at the cervix in HIV-infected women

A number of studies have demonstrated the existence of HIV-specific CD8+ T cells at the cervical mucosa of HIV-infected women (Musey *et al.*, 1997; Kaul *et al.*, 2000; Shacklett *et al.*, 2000a; 2000b; White *et al.*, 2001; Kaul *et al.*, 2003; Musey *et al.*, 2003a; Ibarrondo *et al.*, 2005). The magnitude of *ex vivo* HIV Gag-specific IFN-γ producing CD8+ T cell responses at the genital mucosa in HIV-infected and uninfected women was investigated. Figure 2.2 shows representative plots of IFN-γ production by CD8+ T cells isolated from blood and cervix of an HIV-infected woman.

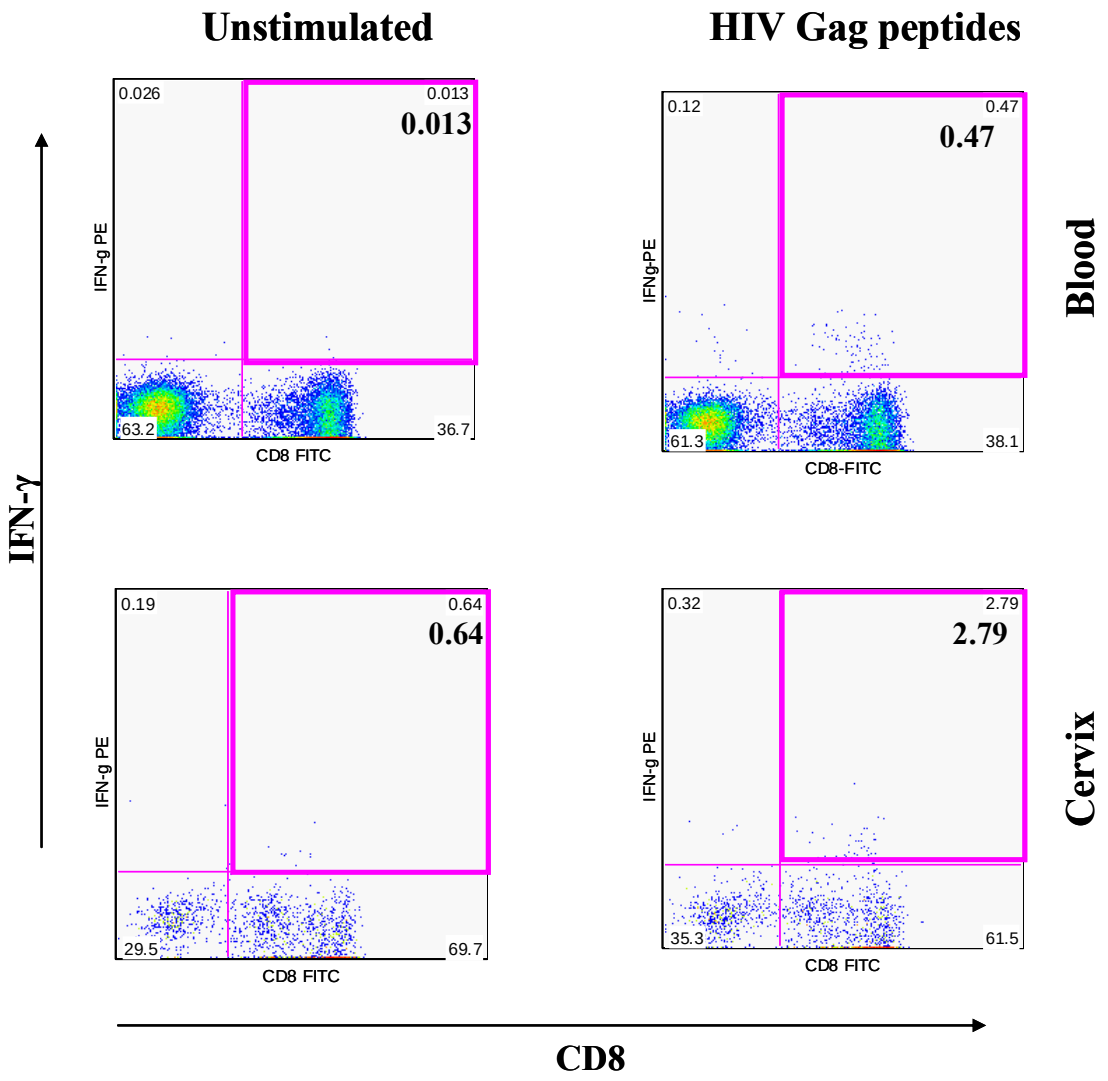
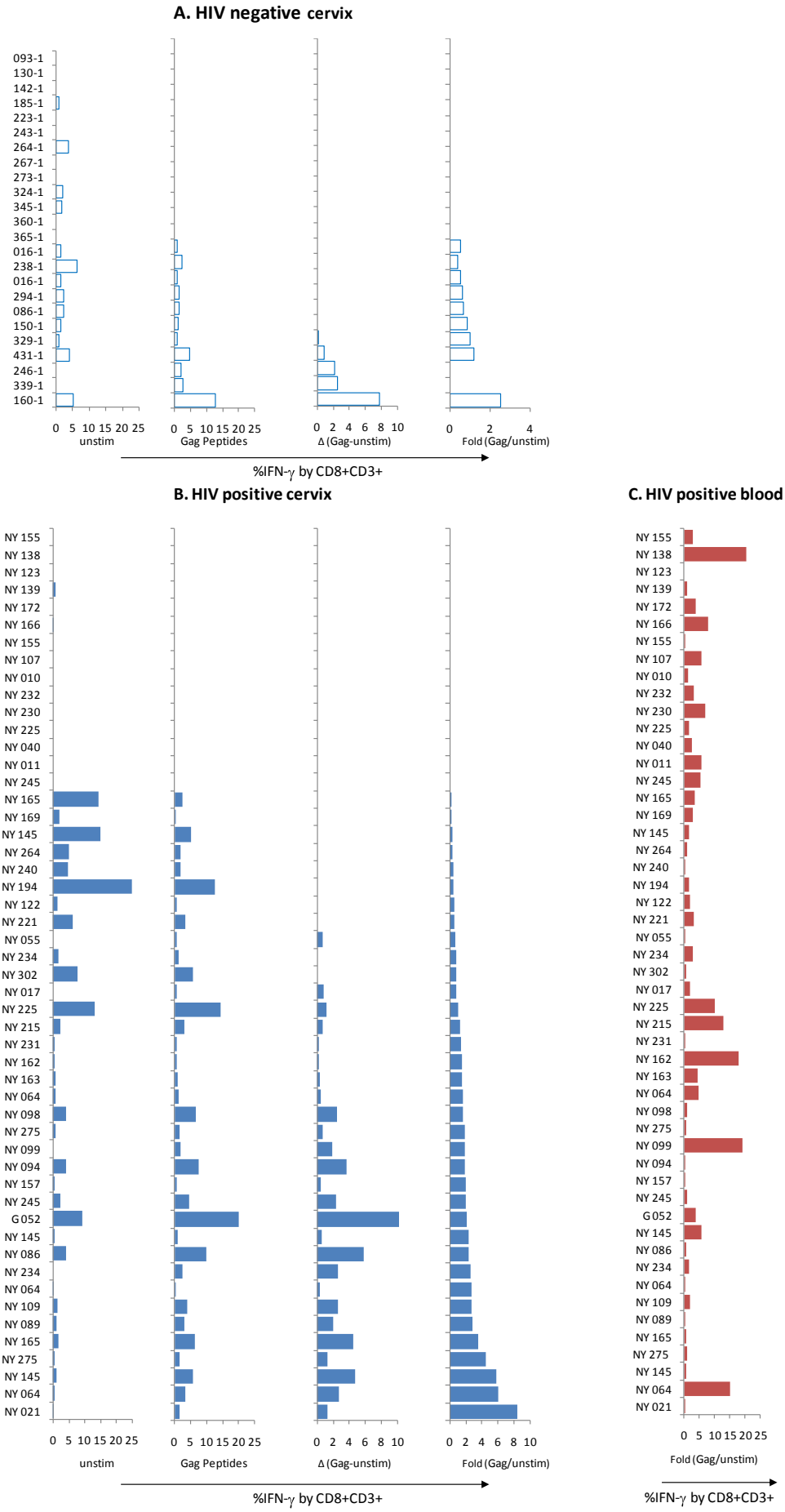


Figure 2.2 Representative plots showing *ex vivo* intracellular IFN-γ production by blood (top panels) and cervical mucosa-derived (bottom panels) CD8+CD3+ cells in response to stimulation with HIV Gag peptides. Cells were either not stimulated (left panels) or stimulated *ex vivo* with a single pool of HIV-1 subtype C Gag peptides for 6 hours (right panels). Events were acquired using a BD FACS Calibur flow cytometer, compensation and analysis was performed using FlowJo software.

From the 81 HIV-infected and 29 uninfected cervical samples analysed, 51/81 and 24/29 samples were SEB or PMA responsive. Only SEB or PMA/ionomycin responsive cervical samples were included in further analysis. The background frequency of IFN-γ producing CD8+ T cells at the cervix (unstimulated) was significantly higher than frequencies observed in matching blood samples [0.65% (0 - 3.63 IQR) background IFN-γ at cervix compared with 0.1% (0.03 - 0.35 IQR) in blood; p=0.001]. There was no significant difference between background

frequencies of IFN-γ+ cells in cervical T cells derived from HIV+ and HIV- women (Figure 2.3A). Both the frequency and fold increase in HIV Gag peptide-specific CD8 responses were significantly higher in chronically HIV-infected women compared to HIV negative women (Figure 2.3A; p=0.04 for % IFN-γ+ to Gag (second panel in A), p=0.02 for net in % IFN-γ+ to Gag (Gag-unstim; third panel in A); and p=0.002 for fold increase in IFN-γ+ responses to Gag (Gag/unstim; forth panel in A). In HIV-infected women, the Gag-specific IFN-γ response frequency by CD8+ T cells ranged from undetectable (0% above background) to 10.8% (above background). Similar analysis in uninfected women showed that Gag-specific IFN-γ responses ranged from undetectable (20/24 participants) to 2.5% (above background). In order to work out an appropriate cut-off for this study, I studied a cohort of 26 HIV negative women from the same community cohort (and with appropriate numbers of CD3+ T cells). Of the 26 HIV negative women included in the study there was one woman that had a much higher Gag-specific IFN-γ response by CD8+ (2.5%, Figure 4.2A) compared to others. These results may indicate that this particular donor is an exposed uninfected women, however, this was not confirmed HIV-specific CTL have previously been identified in cervical cytobrush cells from women who are sexually exposed to HIV but remain HIV seronegative (Kaul *et al*., 2000). Given the level of background and variability in specific response frequencies, it was decided that an appropriate cut-off for positive HIV Gag responses was 3-fold above background which is much more stringent than previous studies. The threshold for positivity was kept consistent between blood and cervix (3-fold).

Figure 2.3 (Over the page) Comparison between *ex vivo* IFN-γ production by cervical mucosa-derived CD8+ T cells from HIV-infected and uninfected women. **A.** Frequencies of IFN-γ+ CD8+CD3+ T cells from cervix in HIV negative women (clear blue bars; n=24). Background frequencies of IFN-γ+ CD8+CD3+ T cells are shown in the first panel. HIV-1 Gag peptide-specific frequencies of IFN-γ+ T cells (without correction for background) are shown in the second panel. Net HIV-1 Gag peptide-specific frequencies of IFN-γ+ T cells (after subtraction of background percentage IFN-γ production by unstimulated cells) are shown in the third panel. Fold increase in IFN-γ production by cervical CD8+ T cells in response to HIV Gag peptides (expressed as a ratio to unstimulated cells) are shown in the fourth panel. **B.** Frequencies of IFN-γ+ CD8+CD3+ T cells from cervix in HIV positive women (solid blue bars; n=51). Background frequencies of IFN-γ+ CD8+CD3+ T cells are shown in the first panel. HIV-1 Gag peptide-specific frequencies of IFN-γ+ T cells (without correction for background) are shown in the second panel. Net HIV-1 Gag peptide-specific frequencies of IFN-γ+ T cells (after subtraction of background percentage IFN-γ production by unstimulated cells) are shown in the third panel. Fold increase in IFN-γ production by cervical CD8+ T cells in response to HIV Gag peptides (expressed as a ratio to unstimulated cells) are shown in the fourth panel. **C.** Fold increase in IFN-γ production by blood CD8+ T cells from HIV-infected women only (solid red bars, n=51) in response to HIV Gag peptides (expressed as a ratio to unstimulated cells). Each bar represents an individual woman's response at the cervix or in blood. The order of the donors (and bars) is the same in each panel. The donors have been stratified according to their hierarchy in fold increase Gag responses at the cervix.


A. HIV negative cervix
093-1
130-1
142-1
185-1
223-1
243-1
264-1
267-1
273-1
324-1
345-1
360-1
365-1
016-1
238-1
016-1
294-1
086-1
150-1
329-1
431-1
246-1
339-1
160-1
0 5 10 15 20 25
unstim
0 5 10 15 20 25
Gag Peptides
0 2 4 6 8 10
Δ (Gag-unstim)
0 2 4
Fold (Gag/unstim)
%IFN-γ by CD8+CD3+
B. HIV positive cervix
NY 155
NY 138
NY 123
NY 139
NY 172
NY 166
NY 155
NY 107
NY 010
NY 232
NY 230
NY 225
NY 040
NY 011
NY 245
NY 165
NY 169
NY 145
NY 264
NY 240
NY 194
NY 122
NY 221
NY 055
NY 234
NY 302
NY 017
NY 225
NY 215
NY 231
NY 162
NY 163
NY 064
NY 098
NY 275
NY 099
NY 094
NY 157
NY 245
G 052
NY 145
NY 086
NY 234
NY 064
NY 109
NY 089
NY 165
NY 275
NY 145
NY 064
NY 021
0 5 10 15 20 25
unstim
0 5 10 15 20 25
Gag Peptides
0 2 4 6 8 10
Δ (Gag-unstim)
0 2 4 6 8 10
Fold (Gag/unstim)
%IFN-γ by CD8+CD3+
C. HIV positive blood
NY 155
NY 138
NY 123
NY 139
NY 172
NY 166
NY 155
NY 107
NY 010
NY 232
NY 230
NY 225
NY 040
NY 011
NY 245
NY 165
NY 169
NY 145
NY 264
NY 240
NY 194
NY 122
NY 221
NY 055
NY 234
NY 302
NY 017
NY 225
NY 215
NY 231
NY 162
NY 163
NY 064
NY 098
NY 275
NY 099
NY 094
NY 157
NY 245
G 052
NY 145
NY 086
NY 234
NY 064
NY 109
NY 089
NY 165
NY 275
NY 145
NY 064
NY 021
0 5 10 15 20 25
Fold (Gag/unstim)
%IFN-γ by CD8+CD3+

2.4.3 Association between the frequency of HIV-specific responses at the cervix and in blood

No correlation was observed between either the net frequency (with background subtracted; data not shown) or fold-increase in IFN-γ production (R=-0.03; p=0.8271; Spearman Rank Test) when blood and cervical T cell responses were compared. This indicates that an HIV-specific response in blood does not necessarily reflect a similar *ex vivo* response at the cervix.

2.4.4 Impact of HIV immune responses at the cervix on HIV shedding at the genital mucosa

Cellular and humoral immune responses against HIV have been detected at the cervix and in semen of both HIV exposed and uninfected (HEPS) and HIV-infected individuals (Mazzoli *et al.*, 1999; Kaul *et al.*, 2000; 2003; Sheth *et al.*, 2005). The detection of these responses in HEPS led to the hypothesis that such responses may be important correlates of protection against HIV locally. However, subsequent studies by Sheth *et al.* (2005) showed that HIV-specific CD8+ T cell responses in semen were not associated with reduced viral shedding but rather significantly associated with semen inflammation. This means that the role of these responses in protection against local HIV shedding is still largely unclear. The relationship between mucosal HIV-specific CD8+ T cells responses and HIV shedding at the genital tract was thus investigated (Figure 2.4). There was no significant association between the presence and magnitude of cervical CD8+ HIV-specific IFN-γ responses and cervical viral load (Figure 2.4A). Similarly in blood, there was no significant association between the magnitude of HIV-specific responses and plasma viral load (Figure 2.4B). Furthermore, women who had undetectable plasma viral load did not have higher magnitude of HIV-responses compared to women with detectable viral loads (data not shown).

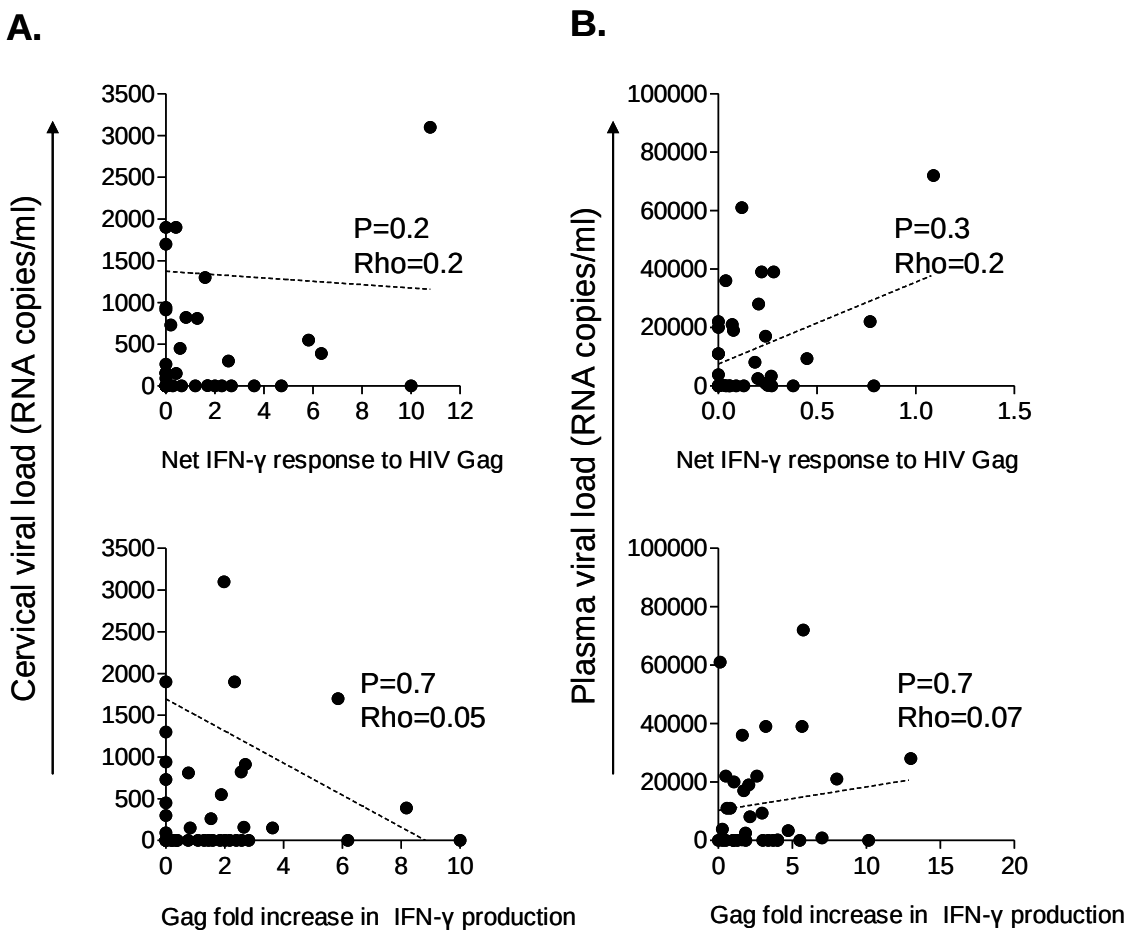


Figure 2.4 Relationship between the magnitude of *ex vivo* HIV-specific IFN-γ production by cervical mucosa- and blood-derived CD8+ T cells and (A) genital HIV-shedding or (B) plasma viral load. (A) Cervical viral load were correlated with either CD8 T cell response magnitude (net resonse), top panel or CD8 T cell response magnitude (fold increase) to Gag peptides, bottom panels. (B) Plasma viral loads were correlated with either CD8 T cell response magnitude (net resonse), top panel or CD8 T cell response magnitude (fold increase) to Gag peptides, bottom panels. Each dot represents an individual women. Spearman Rank test was used to test for correlation.

2.4.5 Cervical inflammation and HIV-specific CD8 T cell responses at the genital mucosa

To investigate whether HIV-specific immunity at the genital mucosa was influenced by genital inflammation, the concentrations of various inflammatory cytokines (IL-12, TNF-α, IL-10, IL-1β, IL-6 and IL-8) at the cervix and in plasma from chronically HIV-infected women was investigated (Table 2.2). Concentrations of TNF-α ($p=0.0025$), IL-10 ($p=0.016$), IL-1β ($p<0.0001$), IL-6 ($p<0.0001$), and IL-8

($p<0.0001$) were found to be significantly elevated at the cervix of HIV-1 chronically infected women compared to matched plasma samples.

Table 2.2 Comparison of inflammatory cytokine levels at the cervix and in blood

Cytokine	Cervix (pg/ml)		Blood (pg/ml)		p-value
	Median	IQR	Median	IQR	
IL-12	6.5	0.0 – 8.6	6.3	0.0 – 7.0	ns
TNF-α	4.4	3.8 – 5.5	3.8	3.5 – 4.0	0.0025
IL-10	4.3	0.0 – 6.3	3.8	0.0 – 4.3	0.0159
IL-1β	42.0	13.0 – 220.8	4.9	4.6 – 5.5	<0.0001
IL-6	25.3	10.3 – 160.7	0.0	0.0 – 5.6	<0.0001
IL-8	1291.2	343.6 – 4305.3	8.1	7.5 – 9.3	<0.0001

The relationship between inflammatory cytokine concentrations at the cervix and in plasma and the magnitude of HIV-specific CD8+ T cell responses at these respective sites was examined (Figure 2.5). There was a significant positive association between the magnitude of cervical HIV-specific CD8+ T cell responses and the levels of TNF-α ($p=0.02$, $r=0.4$), IL-1β ($p=0.05$, $r=0.3$) and IL-6 ($p=0.04$, $r=0.4$) in cervical supernatants (Figure 2.5). This indicates that cervical inflammation was associated with recruitment of HIV-specific T cells to the female genital tract.

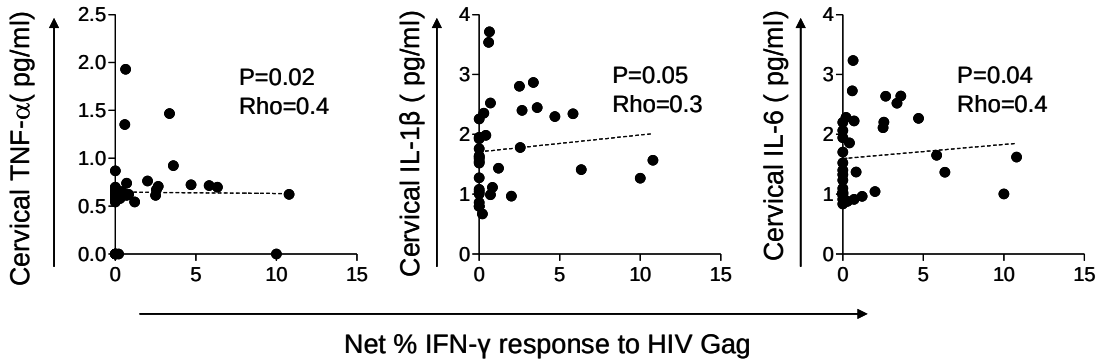


Figure 2.5 Correlation between the concentration of inflammatory cytokines TNF-α, and IL-1β and IL-6 in cervical secretions and the magnitude of HIV Gag peptide-specific IFN-γ responses present at the cervix. Spearman Rank test was used to test for correlation. P<0.05 were considered significant. P- and Rho-values are shown.

2.4.6 Cervical inflammation and HIV shedding at the female genital tract

To investigate whether HIV shedding in the female genital tract was associated with genital inflammation, I compared the concentration of genital inflammatory cytokines in women shedding HIV at the cervix with those who were not (Figure 2.6). Women who were found to be shedding HIV had significantly elevated levels of TNF-α (p=0.026), IL-1β (p=0.0316), IL-6 (p=0.0027) and IL-8 (p=0.0148) compared to women who were not shedding virus (Figure 2.6). In contrast, plasma inflammatory cytokines concentrations were not associated with plasma viral load or cervical shedding (data not shown).

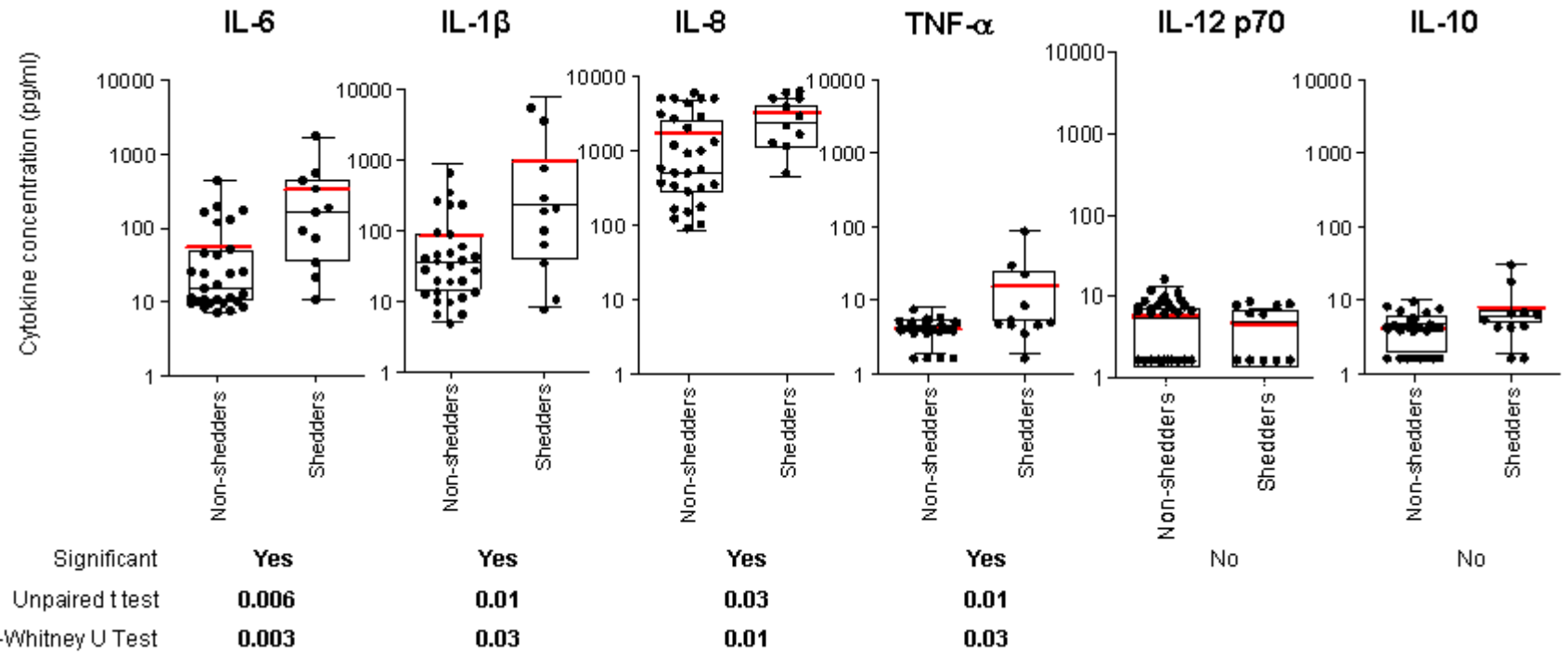


Figure 2.6 Relationship between inflammatory cytokine levels in the cervical supernatant and genital tract HIV shedding in chronically HIV-infected women. Concentrations of IL-6, IL-1β, IL-8, TNF-α, IL-12p70 and IL-10 were measured in cervical secretions from HIV-infected women. Women with detectable cervical viral load (detection limit of 50 RNA copies/ml) were ranked as shedders while women with no detectable cervical viral load were ranked as non-shedders. Each dot represents an individual woman's cytokine concentration in cervical supernatant and actual data has been plotted on a log scale. Horizontal red lines represent mean values for each group. Box-and-Whisker plots represent the median (centre black line), 25th and 75th percentiles concentrations of each inflammatory cytokine measured. P-values ≤ 0.05 were considered significant. The Mann-Whitney U-test was used to compare medians and the students T test was used to compare the means of each groups as indicated.

2.5 Discussion

This Chapter shows that the immunological microenvironment of the female genital tract has a number of features which distinguish it from that found in blood in HIV infected women. The cervical environment is defined by high frequencies of non-specific activation, clearly detectable HIV-specific CD8+ T cells and increased inflammatory cytokine concentrations with the latter feature being associated with HIV shedding. This is the first reported investigation of potential associations between mucosal HIV-specific CD8+ T cell responses, inflammation and HIV shedding in the female genital tract. While an association between either the presence or magnitude of HIV-specific T cell responses at the cervix and local HIV shedding has not been detected, an association between mucosal inflammation and both (i) the magnitude of HIV-specific CD8+ T cell mucosal responses and (ii) local HIV shedding has been shown. These data support the hypothesis that immune activation in the female genital tract is a driver of both local HIV shedding and HIV-specific CD8+ T cell recruitment, and is therefore likely to play a major role in HIV transmission.

A significant positive correlation exists between plasma and cervical viral loads and this Chapter shows that elevated plasma viraemia likely predicts HIV shedding in the female genital tract. Several other studies have reported a positive correlation between plasma viral loads and genital shedding (Speck *et al.*, 1999; Quinn *et al.*, 2000; Bourlet *et al.*, 2001; Sheth *et al.*, 2005; Rebbapragada *et al.*, 2007). Although HIV-1 RNA levels in blood and semen were correlated overall, some participants in the study by Sheth *et al*. (2005) clearly demonstrated disproportionate shedding of HIV-1 RNA in semen. Local factors in the genital tract such as inflammation and sexually transmitted infections are known to be important determinants of HIV shedding in the genital tract (Spear *et al*., 2008). Indeed, CMV reactivation in the genital tract correlated strongly with high semen viral loads, even in the presence of undetectable VL in blood (Sheth *et al*., 2006).

Immune activation and inflammation are crucial features in driving HIV replication and pathogenesis. The findings in this Chapter suggest a generalized activation of the cervix during chronic HIV infection in some women, marked by inflammatory cytokine production. Nkwanyana *et al*. (2009) has shown that genital inflammation is associated with significantly elevated numbers of both CD4+ and CD8+ T cells to the cervix and it is likely that recruitment of activated CD4+ T cells to the cervix during HIV infection would make the female genital tract a "hot-bed" of local viral replication. In support, I show in this Chapter that women shedding HIV in their genital secretions had significantly elevated concentrations of TNF-α, IL-1β, IL-6 and IL-8 in their genital tracts than women not shedding HIV. These data suggest that cervical inflammation is responsible for driving HIV shedding at the cervix or *vice versa*. While several recent studies have shown that ulcerative and inflammatory sexually transmitted infections and resultant inflammation are associated with enhanced HIV-1 shedding (Cummins *et al.*, 2006), the relationship is unclear as others have shown a significant increase in inflammation at mucosal surfaces following HIV-infection and replication (Sankaran *et al.*, 2008).

The finding that elevated systemic inflammation and increased viral loads was not evident in the blood compartment supports the notion that HIV infectiousness may well be related to the level of cervical inflammation in some women. It is known that TNF-α, IL-1β, and IL-6 are pro-inflammatory cytokines associated with enhanced HIV replication (Al-Harthi *et al.*, 1997) and along with IL-8, a chemokine known to attract neutrophils to sites of inflammation (Dinarello, 2000), the cervix is likely to be a hostile activated microenvironment. The association between generalized mucosal cytokine activation and increased mucosal viral loads has previously been observed in the context of HIV-1 infections (Reka *et al.*, 1994; Lawn *et al.*, 2001; McGowan *et al.*, 2004). It may even be argued that exposure of epithelial mucosal barrier to HIV is likely to lead to the secretion of inflammatory cytokines that may damage the epithelial barrier. Recently Nazli *et al*. (2010) showed that direct exposure of intestinal and genital epithelial cells to HIV leads to breaching of the mucosal barrier and increased leakage of both bacteria and virus across the epithelium. The mechanism of this breakdown appeared to be due to inflammatory factors (particularly TNF-α) produced by epithelial cells themselves, in response to HIV-1

exposure, that destroy the tight junctions between epithelial cells, thereby allowing microbial translocation (Nazli *et al.*, 2010).

As in previous studies investigating mucosal HIV-specific CD8+ T cell responses during HIV infection (Musey *et al.*, 1997; Quayle *et al.*, 1998; Kaul *et al.*, 2000; Shacklett *et al.*, 2000a; 2000b; White *et al.*, 2001; Kaul *et al.*, 2003; Musey *et al.*, 2003a; Ibarrondo *et al.*, 2005; Sheth *et al.*, 2005; 2006; Critchfield *et al.*, 2007), it has been shown that mucosal HIV-1 specific CD8+ T cells responses in the female genital tract are directly detectable *ex vivo*. The numbers of cervical mononuclear cells recovered from cervical cytobrush are much lower at the cervix compared to blood. Similar number of matched PBMC could have been used in an attempt of maintaining 'similar' conditions and bias introduced by small number of cells in the assay. A formidable technical hurdle is to discriminate between *in vivo* T cell activation and the presence of HIV-specific immunity. It is also possible that the methodology employed in preparing for intracellular signals may lead to cell exhaustion and death, as these cells are highly activated *in situ*. Bearing this in mind, that no correlation existed between T cell responses detected at the cervix with those in the blood does infer that there may be partial compartmentalization between blood and genital tract due to distinct mucosal environment caused by (i) the localized cytokine milieu, (ii) differing inflammatory signals; and (iii) the presence of different immune cell types in these distinct compartments. In addition, studies done by Musey *et al*., (2003a) found that mucosal and systemic CD8+ CTL clones had similar Env-specific responses and MHC restriction. In light of the fact that the genital tract is not an organized lymphoid structure and is under the influence of local inflammatory signals that regulate cellular recruitment to this site (Nkwanyana *et al*., 2009), the finding that HIV-specific responses between cervical and blood compartments were not associated could be partly due to the above mentioned localized immune factors, leading to shifting of T cell populations in the mucosa.

Although this cross-sectional study cannot prove that HIV-1- specific CD8+ responses in female genital tract increase HIV-1 RNA shedding, these responses were not associated with reduced virus shedding in chronic HIV infection. The use of IFN-γ production as a means of identifying HIV-1-specific CD8+ T cells might bias to

finding an association between some of the inflammatory cytokines and HIV-specific CD8+ responses (Sheth *et al*., 2005). It is becoming clear that relying solely on IFN-γ production by CD8 T cells as a functional readout may not be enough to identify possible correlates of viral control (Pantaleo and Koup 2004; Harari *et al.*, 2006). There is therefore a need for more functional markers such as proliferation, lytic markers, other cytokines and chemokines to be measured in elucidating the role mucosal HIV-specific responses in the prevention of HIV shedding. Broader analysis of functional and phenotypic markers for measuring HIV-specific T cell responses is presented in Chapter 4. It is also possible that cervical HIV-specific CD8 T cells *in vivo* may be present in larger numbers or have enhanced functionality at the mucosa compared with the *ex vivo* analysis performed in this study.

Previous studies of highly exposed but persistently sero-negative (HEPS) women (Kaul *et al.*, 2000) described a relative enrichment of HIV-specific cervical responses in HIV-resistant compared with HIV-infected women and argued that the presence of local mucosal HIV-specific CD8 T cell responses (in the absence of detectable HIV infection) may be important in providing protection at the genital mucosa against HIV infection in these women. The overall magnitude of HIV-specific responses at the cervix of HIV-resistant sex workers was 2-fold lower than the magnitude of responses measured in the HIV-infected cohort (but significantly higher than responses detected at the cervix of HIV-negative women). Since protection from HIV-infection (HEPS study; Kaul *et al.*, 2000) and protection against HIV shedding are different, the responses in the female genital tract of HEPS and HIV-infected women are likely to have different implications in terms of protection, given that the latter have an established chronic infection.

The many differences between the blood and cervical compartments are likely to have an important influence on HIV pathogenesis and transmission. Many biological factors could contribute to the complexity of HIV disease in the female genital tract compartment such as other local immune factors, presence of sexually transmitted infections and hormonal changes over the menstrual cycle. Although this study shows that there is a link between shedding an inflammation, caution should be taken

in the interpretation of these results since this study used the syndromic management approach for sexually transmitted infections (STI). This approach focuses on the detection of symptomatic STIs. Asymptomatic STIs such as BV and HSV-2 which are more common in HIV-infected individuals, may have had effects on both HIV shedding and genital immunology (Rebbapragada *et al*., 2007; 2008).

I found that the magnitude of CD8+ T cells responses detected at the cervix of chronically HIV-infected women correlated significantly with increased concentrations of cervical TNF-α and IL-1β and IL-6. The high level of inflammation (regardless of the cause) in the female genital tract, may contribute to high levels of HIV replication and could result in HIV specific immune response that is responding to increased local levels of HIV but barely effective. While this study and others have identified various factors involved in HIV shedding in the female genital tract, it remains to be determined how these factors interact to influence rates of HIV transmission both between individual sexual partners and at the population level. Unravelling these potentially complex interactions to identify the prime factor(s) driving these associations would be a major advance. It may, for example, be important to determine whether anti-inflammatory interventions might reduce transmission risks in discordant couples. If cervical inflammation is a key driver of HIV shedding in women, then provision of anti-inflammatory preventative or therapeutic treatments – for example in microbicide formulations – may be key in lowering high levels of transmission rates. However, the safety of anti-inflammatory treatments would depend on the cause of the inflammation, since such treatments may blunt the response to other important infections. Clearly, this study highlights the fact that control of inflammation and sexually transmitted infections in the female genital tract should be a major component of HIV prevention programmes. In addition, although this Chapter shows that CD8+ T cells do not correlate with local viral control during HIV infection, this does not preclude such cells containing HIV locally if induced by vaccination prior to HIV infection.

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Chapter 3
Maturational status of T cells derived from the female genital tract during HIV infection

3.1 Summary

Accumulation of highly differentiated T cells in blood is associated with the onset of clinical immunodeficiency in HIV-infected individuals. The impact of HIV infection and clinical stage on the maturational status of T cells derived from female genital tract was investigated. The memory phenotype of cervical cytobrush- and blood-derived T cells from 12 HIV-infected and 36 uninfected women was determined by differential staining of T cells for CD45RA, CD27 and CCR7 and compared with CD4 counts and HIV viral load in blood and in the female genital tract. At the cervix, the frequency of CD4 T cells was reduced in HIV-infected compared to uninfected women ($p<0.0001$) and was associated with the extent of CD4 T cell depletion in blood (Rho=0.6; p=0.03). Effector memory T cells (TEM) predominated at the cervix irrespective of HIV status. The presence of terminally-differentiated T cells (TEff) at the cervix and in blood was significantly higher in HIV-infected compared to uninfected women [cervical CD8 (p=0.05), cervical CD4 (p=0.001); blood CD4 (p=0.02)]. The presence of terminally-differentiated T cells (TEff) at the cervix and in blood was significantly higher in HIV-infected compared to uninfected women [cervical CD8 (p=0.05), CD4 (p=0.001); blood CD4 (p=0.02)]. In HIV-infected women, reduced frequencies of CD4 T cells at the cervix were associated with accumulation of terminally-differentiated cells [CD8 (p=0.03, Rho=-0.6) and CD4 (p=0.002, Rho=-0.7)]. Absolute CD4 counts in blood were inversely correlated with the frequency of TEff cells at the cervix, significantly so for CD8 TEff cells (p=0.05, Rho=-0.61). In HIV negative women, reduced frequencies of CD4 T cells at the cervix were associated with reduced frequencies of cervical central memory CD8 T cells (p=0.0002; Rho=0.6) and increased frequencies of the intermediate CD8 T cells (p=0.01, Rho=-0.4). These data show that low frequencies of cervical CD4 cells were associated with accumulation of mature T cell phenotypes in the female genital tract.

3.2 Introduction

Most pathogens infect humans through mucosal surfaces and the maintenance of memory T cells at these exposed effector sites is important as the first line of defence against pathogenic invasion (Hayday *et al.*, 2001; Nagler-Anderson, 2001; Masopust *et al.*, 2006). While the mucosal surfaces of the female genital tract serve as the major portal of entry for HIV during heterosexual transmission, the mucosal surface of the gut serve as the predominant site of viral replication and CD4 T cell depletion (Johnson and Kaur, 2005; Li *et al*., 2005; Mattapallil *et al*., 2005). The female genital tract is a tertiary effector site which lacks organized lymphoid structures (Wu *et al*., 2000; Russell and Mestecky, 2002) and immune cells residing here are thought to be recruited in response to an inflammatory signal (Lebre *et al*., 2005; Nagarajan *et al*., 2008; Nkwanyana *et al*., 2009) in an integrin dependent manner (Hawkins *et al*., 2000; Cicala *et al*., 2009). The presence of T cells with the ability to respond rapidly at mucosal epithelial surfaces is essential as these cells allow for rapid containment of invading pathogens at the local entry sites and prevent systemic spreading (Cheroutre and Madakamukil, 2005)

HIV is a chronic viral infection which has been associated with gradual exhaustion of the T cell memory pool (Farrel, 2006; El-Far *et al*., 2008). Throughout the course of HIV infection, there are alterations in the phenotypic and maturational characteristics of T cells, reflected in accumulation of terminally-differentiated T cells during late stage disease (Appay and Rowland-Jones, 2004). A better understanding of this process of T cell differentiation and maturation and its role in viral control is important for our understanding of T-cell mediated immunity. Studies of the maturational status of immune cells present in the female genital tract may give important insight into events associated with HIV transmission (Poonia *et al*., 2006b).

T cells can be divided into distinct memory subsets which differ in their homing capacity, and their ability to proliferate and produce cytokines in response to stimuli (Sallusto *et al.*, 1999). Naïve T cells (Tn) have never come in contact with their cognate antigen, are characterised by expression of CD45RA, the lymph node homing receptors CCR7 and CD62L, co-stimulatory receptors CD28 and CD27, low expression of the integrin CD11a, and lack of expression of markers such as CD57, granzymes and perforin (Appay and Rowland-Jones,

2004). The ontogeny of memory T cells is still being debated with different studies proposing a linear differentiation pathway of T cells and others suggesting a complex differentiation pathway (Ahmed and Gray, 1996; Sallusto *et al*., 1999; Champagne *et al*., 2001; Kaech *et al*., 2002; Seder and Ahmed, 2003; Tussey *et al*., 2003; Wherry *et al*., 2003; Appay and Rowland-Jones, 2004; Lefrancois and Marzo, 2006; Ahmed *et al*., 2009).

Compared with Tn cells, memory T cells are more rapidly dividing, express adhesion molecules that facilitate extravasation to tissues and express the low-molecular-weight isoform of CD45 (CD45RO; Connors *et al*., 1997). Memory T cells can be further divided into distinct memory subsets based on the expression of the CCR7, CD62L, CD27, CD28 and CD45RA. These memory T cell subsets differ in their proliferative capacity and cytokine production profiles (Sallusto *et al*., 1999; Tilton *et al*., 2007). Central memory T cells (TCM) express CCR7 but not CD45RA, preferentially circulate to lymph nodes, produce IL-2 upon encounter with antigen, possess high self-renewal ability and express several anti-apoptotic genes associated with long-term survival (Halwani *et al*., 2006). Effector memory T cells (TEM), on the other hand, do not express CCR7 or CD45RA, circulate to tissues in response to inflammation, and have potent effector functions upon antigen encounter (Sallusto *et al*., 1999; Halwani *et al*., 2006). TEM cells are more short-lived compared to TCM and the molecular basis for this is in their expression of several pro-apoptotic genes (Harari *et al*., 2004; Riou *et al*., 2007; Tilton *et al*., 2007).

A class of terminally-differentiated effector T cells (TEff), distinct from TEMs, has also been proposed which do not express CCR7 but have regained expression of CD45RA (Yue *et al*., 2004; Tilton *et al*, 2007). Furthermore, an intermediate stage of T cell differentiation has been demonstrated in T cells (TInter) in studies that have also included the co-stimulatory molecule CD27 as a phenotypic marker (Wills *et al*., 2002). TInter cells express CD45RA and CD27 but do not express CCR7 (Appay *et al*., 2002; Wills *et al*., 2002). Transitional memory cells (TTM) are thought to have dual differentiation potential. Depending on the level of antigenic stimulation, they can either revert towards a TCM phenotype or differentiate towards a TEM phenotype (Verhoeven *et al*., 2008). These T-cell populations can be grouped further into “early”, “intermediate” and “late” subsets based on their position along a linear pathway of longevity and expression of CD127 on long-lived and CD57 on short-lived T cells: Tn cells (CD45RA+CCR7+CD27+)→ “early” TCM (CD45RA-CCR7+CD27+)→ “intermediate” transitional memory cells (TTM; CD45RA-CCR7-

CD27+)→ TInter (CD45RA+CCR7-CD27+)→ "late" TEM (CD45RA-CCR7-CD27-)→ terminally-differentiated TEff cells (CD45RA+CCR7-CD27-; Appay and Rowland-Jones, 2004; Sauce *et al*., 2007; Burgers *et al*., 2009).

Studies during acute HIV and SIV infections have shown that CD4 TEM cells are rapidly depleted at mucosal effector sites (GALT and lungs) while the TCM pool is largely preserved (Picker *et al.*, 2004; Grossman *et al.*, 2006; Picker, 2006; Okoye et al, 2007). About 30%-60% of these mucosal CD4 TEM cells are destroyed in the first few days of HIV infection (Li *et al*., 2005; Mattapallil *et al*., 2005). During chronic HIV infection, several studies have shown that maintenance of TEM numbers is dependent on the preservation of TCM cells for their homeostasis and this TCM-dependent TEM production progressively declines during chronic infection (Marzo *et al.*, 2007; Okoye *et al.*, 2007). The frequency of CD4 TCM cells in GALT was found to be associated with better mucosal CD4 T -cell restoration (Verhoeven *et al*., 2008). Despite replenishing of the TEM pool by TCM cells, the frequency of CD4 cells remain low at the mucosal sites during chronic HIV infection compared to uninfected individuals (Tedla *et al*., 1999; Critchfield *et al*., 2008).

Previous studies have demonstrated that the differentiation status of T cells influences the rate of HIV disease progression (Papagno *et al.*, 2004; Potter *et al*., 2007; Ladell *et al*., 2008; van Grevenynghe *et al*., 2008; Burgers et al., 2009). Burgers *et al.* (2009) showed that higher frequencies of early and intermediate CD8 memory cells during early HIV infection were associated with subsequently lower viral set points. Papagno *et al.* (2004) reported an inverse correlation between CD4 T cell count and percentage of highly differentiated CD27- cells in the whole CD8+ T cell population of HIV-1 infected donors during chronic infection. Further, individuals who are able to better control HIV infection have a preserved TCM pool, (Potter *et al.*, 2007; van Grevenynghe *et al*., 2008), while individuals with a reduced TCM CD4 pool had increased plasma HIV viral load (Ladell *et al*., 2008).

The aim of this study was to investigate the impact of HIV infection and clinical status on the maturational status of T cells derived from female genital tract and blood. T cells in the genital tract in the absence of HIV infection and during chronic HIV infection were characterized by differential staining of T cells for CD45RA, CD27 and CCR7.

3.3 Methods

3.3.1 Description of individuals included in the study

Thirty eight chronically HIV-infected and 62 uninfected women attending the Manyanani Emphilisweni community clinic in Gugulethu, Cape Town were enrolled in this study. Women who were menstruating at the time of sampling, who were post-menopausal or had undergone a hysterectomy were excluded from the study. The syndromic management approach for STI which is used in many resource-poor settings and advocated by the World Health Organisation (WHO) was used in this study. This approach is based upon the concept of covering all likely pathogens responsible for a number of clinical syndromes (Lewis *et al*., 2008). In this study, a thorough genital examination for vaginal discharge, visible ulcers or genital warts was done. Women who were found to have any of these symptoms were excluded. This approach, however, did not take into account asymptomatic infections. The study was approved by the Research Ethics Committee of the University of Cape Town, South Africa (UCT REC 258/2006) and informed written consent was obtained from all volunteers of the study.

3.3.2 Cervical cytobrush collection and processing

Cervical mucosal mononuclear cells were collected using a Digene cervical sampler and processed as previously described in Chapter 2 (Section 2.3.2).

3.3.3 Counting of cervical cells using a Guava cell counter

Cervical cytobrush-derived cells were counted using a Guava Automated cell counter to quantify fluorescently labelled CD3 T cells as previously described in Chapter 2 (Section 2.3.4).

3.3.4 Peripheral blood mononuclear cells (PBMCs) isolation

Blood from HIV-infected and uninfected women were collected using standard venipunture and PBMCs were isolated using Ficoll-Hypaque (Sigma-Aldrich, Egham, Runnymede, UK) density gradient centrifugation as described in Chapter 2 (Section 2.3.5).

3.3.5 Counting of PBMCs using an automated Guava cell counter

PBMCs were counted on a Guava Automated cell counter using Guava Viacount reagent (Guava technologies, Hayward, CA) and analyzed using Cytosoft® software (Guava technologies, Hayward, CA) as described in Chapter 2 (Section 2.3.6).

3.3.6 Flow cytometry

CD8 and CD4 cervical cytobrush- and blood-derived CD3 T cells were evaluated for cell surface expression of maturational markers CD45RA, CD27 and CCR7. Cervical cells (0.1-1×10^6 cell/ml) and PBMC (1×10^6 cell/ml) were stained with pre-titrated phenotypic markers Pacific blue-labelled anti-CD3 (BD Biosciences), PercPCy5.5-labelled anti-CD8 (BD Biosciences), APC-labelled anti-CCR7 (R&D Systems Inc., Minneapolis, MN), PE-labelled anti-CD27 (BD Biosciences) and Cy7.PE-labelled anti-CD45RA (BD Biosciences) for 1 hour at 4°C. Cells were washed with 2 ml of 10% FCS PBS (0.01% NaN_3), centrifuged (5 minutes, 300×g, 1300 rpm, 4°C) and fixed with BD Cell Fix. Cell fluorescence was measured by flow cytometry a LSRII flow cytometer (BD Biosciences). FlowJo version 8.5.3 (Tree Star, Inc; Ashland, Oregon, OR, USA) was used to set compensation and for data analysis. Fluorescence minus one (FMOs) were used to set gates. In this study, CD8 T cells were defined as CD3 cells expressing CD8. CD4 was not measured directly in this study and CD4 T cells were defined as CD3 cells not expressing CD8. Studies from our laboratory (Nkwanyana *et al*., 2009) and others (Kallas *et al.*, 1999) have shown that ≥95% of CD3 T cells not expressing CD8 expressed CD4.

3.3.7 Determination of viral load in cervical supernatant and plasma

HIV load was determined in cervical secretions and plasma from HIV-infected women using Nuclisens Easyq HIV 1 Version 1.2 by the NHLS Diagnostic Virology Laboratory (Groote Schuur Hospital, Cape Town, South Africa) as described in detail in Chapter 2 (Section 2.3.9). The detection limit of this assay was ≥50 HIV RNA copies/ml. Values below the assay's detection limit were reported as zero.

3.3.8 Statistical analysis

Where indicated, Mann–Whitney U test was used for non-parametric comparisons. Spearman Rank test was used to test for correlations using GraphPad Prism version 5.0. All tests were two-tailed and P-values ≤ 0.05 were considered significant.

3.4 Results

Thirty eight HIV-infected and 62 uninfected women were enrolled in this study to investigate the impact of HIV infection and clinical status on the maturational status of T cells derived from the female genital tract. I used a 7-colour staining panel which included CD3, CD8, CD45RA, CCR7, CD27, IFN-γ and IL-2 (cytokine expression data was not reported in Chapter 3) The limitation of this panel was that it did not include a viability marker. Although this chapter looks at the maturational status of unstimulated cells, I used IFN-γ responsiveness to PMA/ionomycin (positive control) in cervical cytobrush samples as an inclusion criterion for "viable" samples. In our laboratory, we found that *ex vivo* CD3 cell counts in cervical cytobrush samples correlated significantly with the frequency of T cells producing IFN-γ following stimulation with PMA/ionomycin (Rho=0.5, P<0.0001; Liebenberg *et al*., manuscript submitted). Furthermore, cervical samples which failed to respond to PMA/ionomycin had significantly lower CD3+ events (median less than $\leq 10^4$, as indicated by the number of CD3+ events counted by guava cell counter) than cytobrush samples that yielded positive IFN-γ responses to PMA/ionomycin. From this finding, samples with CD3+ event counts $\leq 10^4$ or were unresponsive to PMA/ionomycin were excluded from further analysis.

Of the 62 cervical cytobrush samples obtained from uninfected women, 20/62 (32.3%) yielded too few cells for flow cytometic analysis and 6/62 (9.7%) failed to respond to PMA/ionomycin. Of the 38 cervical samples from HIV-infected women, 17/38 (44.7%) were excluded due to low CD3 yield while 9/38 (23.7%) failed to produce IFN-γ in response to PMA/Ionomycin. The frequency of cervical specimen exclusions due to low cell numbers or PMA failure did not differ significantly in HIV-infected and uninfected women (p=0.2 for low cell number and p=0.06 for PMA failures, X^2 test).

Of the 38 HIV-infected and 62 uninfected women enrolled, cervical cytobrush samples from 12/38 (31.5%) of HIV-infected and 36/62 (58.1%) of uninfected women were suitable for inclusion and there was a significant difference between the number of eligible samples between groups (p=0.01; X^2 test). No differences were found in the clinical status (CD4 counts or plasma viral loads) of the HIV-infected women who were excluded versus included from this study (data not shown).

3.4.1 Clinical description of study participants

Twelve HIV-infected and 36 uninfected women were included in this study to investigate the impact of HIV infection and clinical status on the maturational status of T cells derived from the female genital tract (Table 1). All HIV-infected women were naïve to anti-retroviral therapy at the time of study. The median age of HIV-infected women was 35.0 years [Inter-quartile range (IQR) 28-43] and the median age of the uninfected women was 35.5 years (IQR 30-47). The median CD4 T cell count of HIV-infected women was 292 cell/µl (IQR 142-559 cell/µl) and median plasma viral load was 3000 RNA copies/ml (IQR 180-31000 copies/ml). The median viral load detected in cervical secretions was 200 RNA copies/ml (IQR 0-540) and this was significantly lower than that detected in plasma (p=0.04). As reported in Chapter 2, there was a significant positive association between the amount of HIV being shed in the genital tract and plasma viral load (Rho=0.69; p=0.02). The ratio of CD4:CD8 T cells derived from cervical cytobrushes from HIV-infected women was significantly lower than that found in uninfected women [median CD4:CD8 of 0.58 (IQR 0.29-0.85) for HIV+ versus 2.17 (IQR 1.29-3.44) for uninfected women; p<0.0001] reflecting depletion of CD4 T cells in the genital tract during HIV infection.

Table 3.1 Clinical characteristics of HIV-infected and uninfected women

Characteristics	HIV-infected	Uninfected
N	12	36
Age in years median (IQR)	35.0 (28-43)	35.5 (30-47)
Absolute blood CD4 count [cells/μl; median (IQR)]	292 (142-559)	nd
Cervical CD4:CD8 ratio median (IQR)	0.58 (0.29-0.85)*	2.17 (1.29-3.44)
Cervical viral load [RNA copies/ml; median (IQR)]	200(0-540)	nd
Plasma viral load [RNA copies/ml; median (IQR)]	3000 (180-31000)	nd
Number of women with detectable plasma viral load (N; %)	9/11[b] (81.8%)	nd
viral load in women with detectable levels [RNA copies/ml, median (IQR)]	22000 (1675-32500)	nd
Range of plasma viral load in women with detectable levels (RNA copies/ml)	180 – 270000	nd
Number of women with detectable cervical viral load (N; %)	8/12 (66.7%)	nd
Cervical viral load in women with detectable levels [RNA copies/ml, median (IQR)]	390 (155-1268)	nd
Range of cervical viral load in women with detectable levels (RNA copies/ml)	82–3300	nd

*The ratio of cervical CD4:CD8 T cells HIV-infected women was significantly lower than in uninfected women ($p<0.0001$)
[b]Plasma samples were only available for viral load assessment from 11/12 of the HIV-infected women

3.4.2 CD4 depletion in the female genital tract mirrors blood during HIV infection

Gut-associated mucosal surfaces are major sites of CD4 T cell depletion during HIV infection (Mattapallil *et al*., 2005; Li *et al*., 2005). To evaluate whether CD4 T cells are similarly depleted in the female genital tract during HIV infection, cervical and blood CD4 T cell frequencies (expressed as a percentage of CD3 T cells) were compared in chronically HIV-infected and uninfected women (Figure 3.1). As expected, CD4 percentages in blood were significantly reduced in HIV-infected compared to uninfected women ($p=0.0002$). Similarly, CD4 T cells at the cervix were significantly reduced in HIV-infected women compared to uninfected women ($p<0.0001$) (Figure 3.1A). There was significant correlation between the percentage CD4 T cells at the cervix and blood in HIV-infected women indicating the extent of CD4 depletion was related between compartments (Figure 3.1B, $p=0.03$, $Rho=0.62$). The proportions of CD4 T cells were not significantly different at the cervix compared to blood, indicating that the female genital did not have higher levels of CD4 depletion than blood.

The association between cervical and blood CD4 percentages was not observed in uninfected women (data not shown).

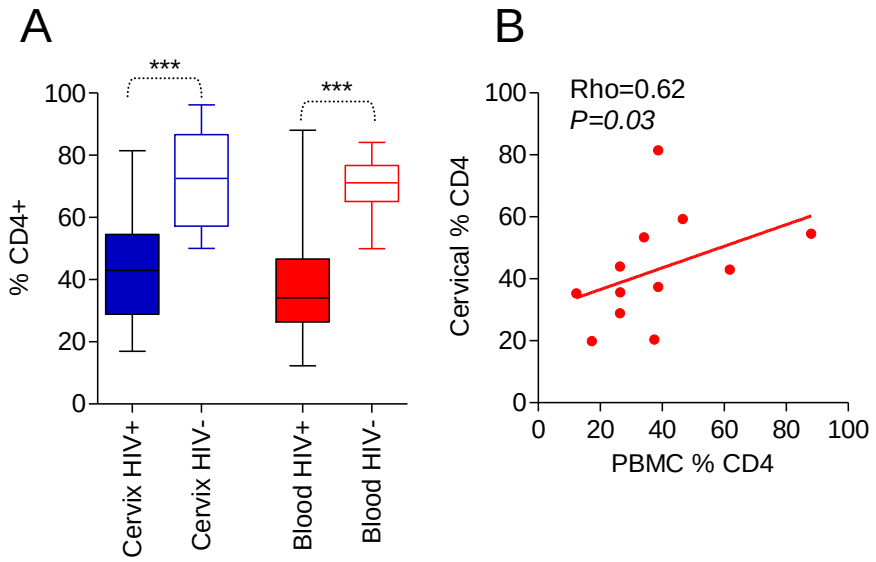

Figure 3.1 CD4 percentages at the cervix and in blood of HIV-infected and uninfected women. (**A)** CD4 percentages (expressed as percentage of total of CD3 T cells) in cervical mucosa (blue boxes) and blood (red boxes). Horizontal lines within each box represent the median while the outer limits of the box indicate the 25th and 75th percentiles. The whiskers indicate the 5-95th percentile. Asterisks indicate significant differences between HIV-infected and uninfected women (***p<0.001). (**B)** Association between cervical and blood CD4 T cell percentages in HIV infected women. Spearman rank test was used to test the association. P-values ≤0.05 were considered significant.

3.4.3 Definition of distinct T cell memory subsets

The maturational status of T cells in the female genital tract and in blood was defined in this study based on differential staining with CD45RA, CD27 and CCR7. Using these markers, six distinct T cell memory subsets were defined (Table 3.2): (1) Tn (CD45RA+CCR7+CD27+), (2) TCM (CD45RA-CCR7+CD27+), (3) TTM (CD45RA-CCR7-CD27+), (4) TInter (CD45RA+CCR7-CD27+), (5) TEM (CD45RA-CCR7-CD27-), and (6) terminally-differentiated TEff cells (CD45RA+CCR7-CD27-). Tn and TCM subsets were considered to be the long-lived cells. TInter and TTM subsets were considered to be at an intermediate stage of differentiation. TEM and TEff subsets were considered to be short-lived and in the late stages of differentiation. Figure 3.2 shows the gating strategy used to define these T cell subsets based on CD45RA, CCR7 and CD27 in blood and at the cervix.

Table 3.2 T cell memory subsets defined by expression of CD45RA, CCR7 and CD27

Memory T cell subset	Longevity	Abbreviation	Memory markers		
			CD45RA	CD27	CCR7
Naïve	Long-lived	Tn	+	+	+
Central memory	Long-lived	TCM	-	+	+
Transitional memory	Intermediate	TTM	-	+	-
Intermediate	Intermediate	TInter	+	+	-
Effector memory	Short-lived	TEM	-	-	-
Terminally differentiated effectors	Short-lived	TEff	+	-	-

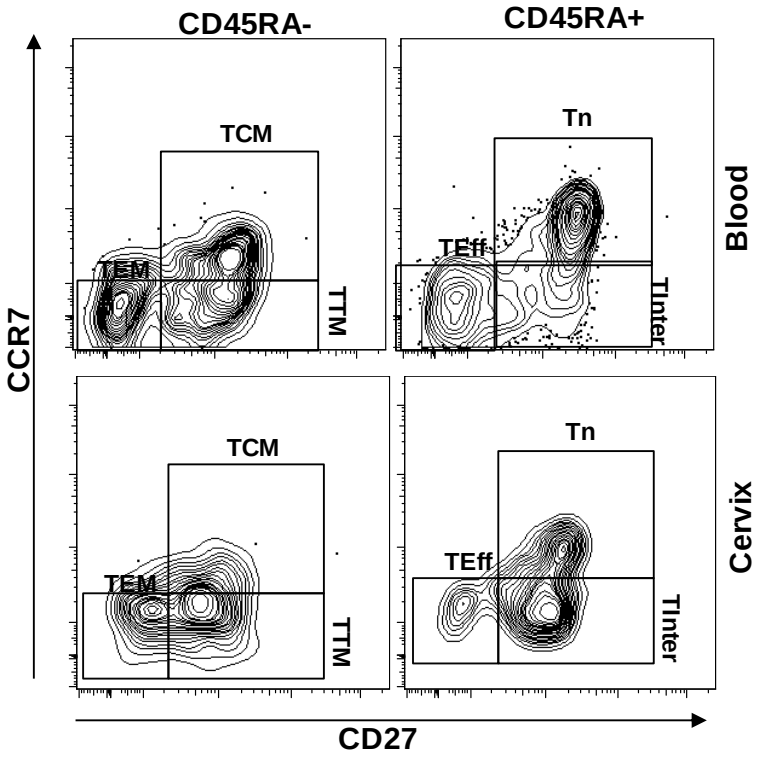

Figure 3.2 Representative plots showing the gating strategy used to define memory subsets of blood and cervical T cells based on expression of CD45RA, CCR7 and CD27. T cells were defined as CD45RA-CCR7+CD27+ TCM, CD45RA-CCR7-CD27+ TTM, CD45RA-CCR7-CD27- TEM, CD45RA+CCR7+CD27+ Tn, CD45RA+CCR7-CD27+ TInter; and CD45RA+CCR7-CD27- terminally-differentiated TEff cells. FlowJo version 8.5.3 was used to set compensation and for analysis. FMOs were used to set gates.

3.4.4 Distribution of T-cell differentiation subsets in the female genital tract and blood

To investigate whether tissue location has an impact on the distribution of T cell memory subsets, the distribution of CD8 and CD4 T cell memory subsets at the cervix and in blood were compared (Figure 3.3). Irrespective of HIV status and T cell subset, TEM cells were the predominant T cell subset identified at the cervix making up ~40% of T cells isolated by cytobrush and the frequency of TEM cells were significantly higher than detected in blood ($p<0.0001$ for HIV-CD8, $p=0.02$ for HIV+CD8; $p=0.0002$ for HIV-CD4 and $p=0.001$ for HIV+CD4 subset). In contrast, terminally differentiated TEff were present at a lower proportion in both CD8 and CD4 subsets at the cervix compared to blood ($p<0.0001$ for HIV-CD8; $p=0.04$ for HIV+CD8; $p=0.03$ for HIV-CD4 and $p=0.0002$ for HIV+CD4).

CD8 and CD4 Tn cells, CD4 TInter and CD8 TCM subsets represented only minor populations of T cells at the cervix. The frequencies of CD8 and CD4 TCM, however, were elevated at the cervix than in blood in both HIV-infected and uninfected women, significantly so for uninfected women ($p=0.0001$ for CD8 and $p=0.02$ for CD4). Similarly, both CD4 and CD8 TInter cells were significantly reduced at the cervix compared to blood irrespective of HIV status ($p<0.0001$ for HIV-CD8; $p=0.01$ for HIV+CD8; $p<0.0001$ for HIV-CD4 and $p=0.0005$ for HIV+CD4). TTM cells were present at similar proportion in cervical and blood compartments, except for CD4 TTM in blood of uninfected women which was present at a significantly higher frequency compared to cervical compartment ($p<0.0001$).

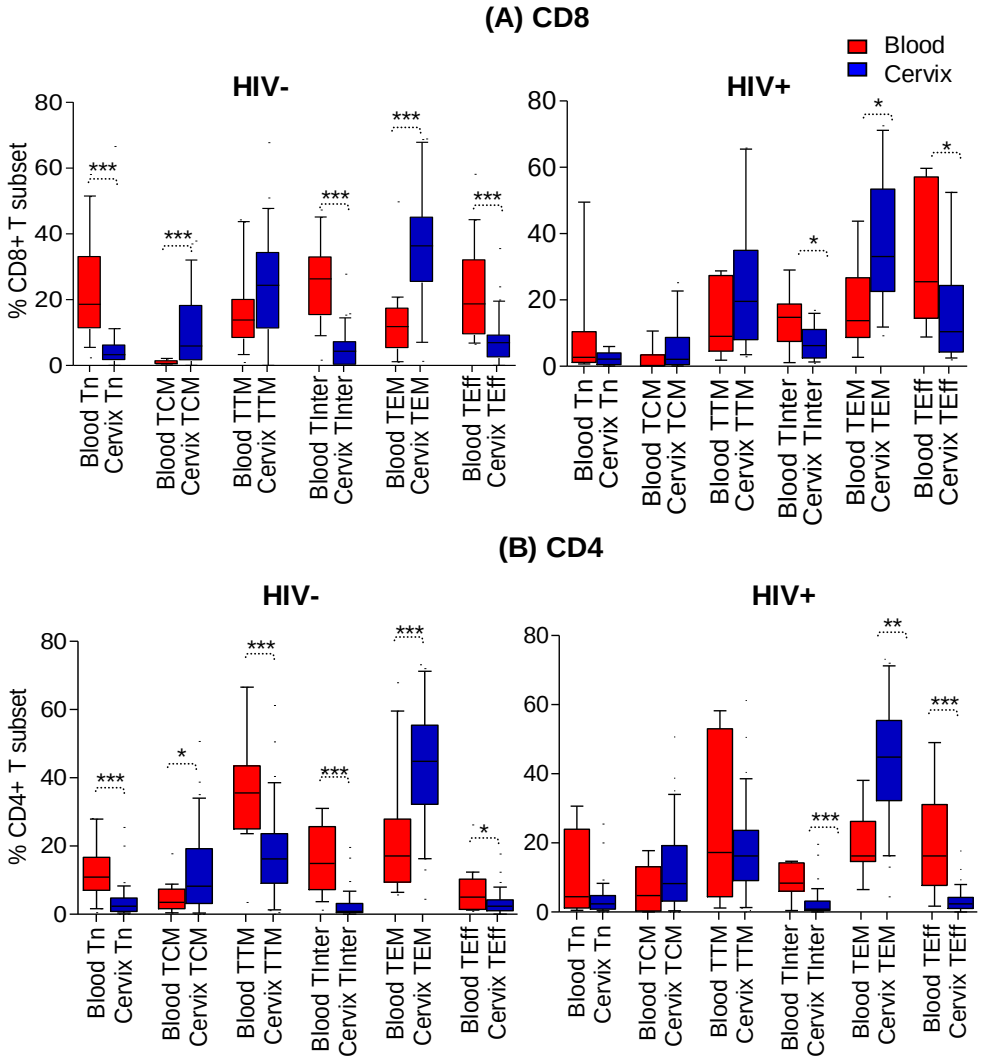

Figure 3.3 Comparison of the frequency of distinct T cell memory subsets found at the cervix and in blood. Frequencies of Tn, TCM, TTM, TInter, TEM and TEff CD8 T cells (A) and CD4 T cells (B) in blood (red boxes) versus cervix (blue boxes) from uninfected women (left panels) and HIV-infected women (right panels). Frequencies represent the percentage of each subset in total CD8 or CD4 T cells. Horizontal lines represent medians. Asterisks indicate significant differences between blood and cervix (*p<0.05; **p<0.005 and ***p<0.001).

3.4.5 Impact of HIV infection on differentiation status of T cells at the cervix and in blood

Premature aging in memory phenotypes of T cells in blood as a result of HIV infection have previously been linked to HIV disease progression (Cao *et al.*, 2009). To investigate whether HIV infection impacts on T cell maturation at the female genital tract, the distribution of T cell memory subsets in the genital tract were compared in HIV-infected and uninfected women (Figure 3.4). HIV status did not impact on the frequency of TEM cells present at the

cervix or in blood. HIV-infected women had higher frequencies of TEff at the cervix and in blood (at the cervix p=0.05 for CD8; p=0.001 for CD4 and in blood p=0.02 for CD4). Although present at very low frequencies, HIV-infected women had significantly lower frequencies of blood CD8 Tn (p=0.01; Hazenberg *et al.*, 2000), CD8 TInter (p=0.03), and CD4 TInter (p=0.05) cells than uninfected women.

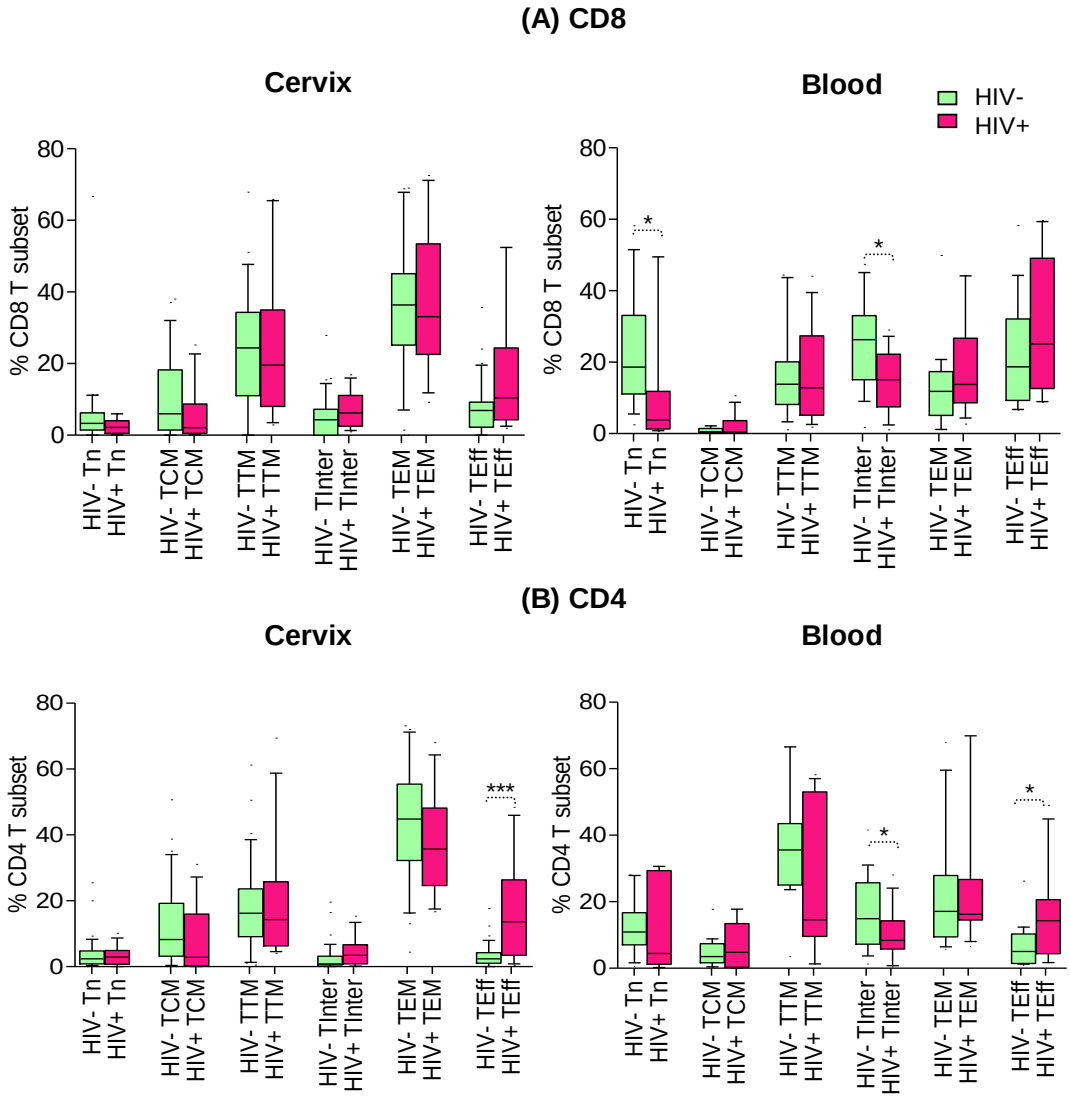

Figure 3.4 Impact of HIV infection on differentiation status of T cells derived from the cervix and blood. Memory subset distribution by CD8 (A) and CD4 (B) T cells isolated from the cervix (left panels) and blood (right panels) of HIV- (green boxes) and HIV+ women (pink boxes). Frequencies displayed represent the percentage of each subset in total CD8 (left panels) or CD4 T cells (right panels). Horizontal lines within boxes represent medians. Asterisks indicate significant differences between HIV negative and positive women (*p<0.05; ***p<0.001).

3.4.6 Association between HIV clinical status and memory phenotype of T cells in the female genital tract and blood of HIV-infected women

Our laboratory has recently shown that HIV-infected women have significantly lower CD4:CD8 ratios in the genital tract than uninfected women indicative of CD4 depletion (Nkwanyana *et al.*, 2009). Consistent with this, HIV-infected women included in this study were also found to have significantly lower median CD4:CD8 ratios at the cervix than uninfected women ($p<0.0001$; data not shown). Papagno *et al*. (2004) previously found a negative association between CD4 counts in blood during chronic HIV infection and the percentage of terminally-differentiated CD8 T cells, suggesting that HIV disease progression is associated with accumulation of T cells with a more exhausted phenotype. The impact of HIV clinical status (absolute CD4 counts in blood and viral load) on the frequency of memory T cell subsets at the cervix and in blood was investigated in HIV-infected women.

Frequency of CD8 TTM cells at the cervix were significantly positively associated with absolute CD4 counts in blood ($p=0.04$, Rho=0.6). Conversely frequency of CD8 TEff cells at the cervix was significantly negatively associated with absolute CD4 counts in blood ($p=0.04$, Rho=0.6) (Figure 3.5). .In contrast, no association between the differentiation status of T cells in the female genital tract and plasma or cervical HIV viral loads was observed (data not shown). These findings suggest that HIV does not directly drive terminal differentiation of T cells in the female genital tract, but that other immune factors such as immune activation or inflammation may be driving the differentiation of T cells observed in women with advanced HIV infection. No significant association was observed between blood T cell memory phenotype and HIV clinical status (data not shown).

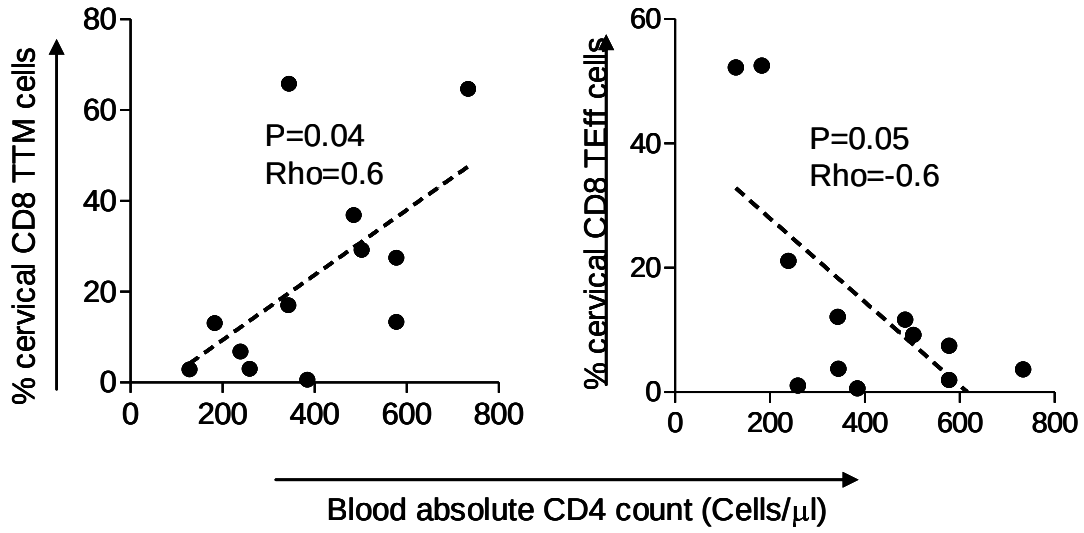

Figure 3.5 Association between blood absolute CD4 counts and the frequency of CD8 memory phenotype of T cells in the female genital tract of HIV-infected women. Frequency (percentage of CD3+ CD8 T cells) of cervical CD8 TTM and TEff cells were correlated to absolute CD4 T cells in blood Each dot represents an individual woman's proportions. Dotted lines represent best fits as predicted by linear regression. Spearman rank test was used to test the correlation. P≤0.05 were considered significant.

3.4.7 Relationship between the frequency of CD4 T cells at the cervix and differentiation status of T cells in the female genital tract

Since it was found that HIV clinical status impacted significantly on T cell memory subset distribution in the genital tract, we wanted to investigate whether this was restricted to HIV infection or a more general phenomenon associated with the female genital mucosa. The relationship between cervical CD4 frequency and the frequency of long-lived (Tn and TCM), intermediate (TTM and TInter) and short-lived (TEM and TEff) T cell subsets detected in the genital tract in all women studied was evaluated by logistical regression analysis (Figure 3.6). .Both CD8 and CD4 Tn, TTM and TEM subsets did not have any significant association with CD4 frequencies at the cervix in all women (data not shown).

In HIV-uninfected women, low CD4 frequencies were significantly correlated with reduced frequencies of CD8 T cells that are long-lived [CD8 TCM (p=0.0002; Rho=0.6); Figure 3.6A]; and elevated frequencies of CD8 T cells in the intermediate stages of differentiation [CD8 TInter (p=0.011, Rho=-0.4) Figure 3.6C]. Although similar trend was observed in TCM and Tinter CD4 T cells their association with frequency of cervical CD4 T cells was not significant (Figure 3.6B and Figure 3.6D). In HIV-infected women, CD4 frequencies at the cervix had significant negative correlation with cervical CD8 and CD4 T cells in the late

stages of differentiation [CD8 TEff (p=0.0005, Rho=-0.5; Figure 3.6E; CD4 TEff (p=0.0007, Rho=-0.5), Figure 3.6F, also shown in Table 2]. In general, this data indicates that HIV negative women with reduced frequencies of CD4 T cells in their genital tracts are likely to have reduced frequencies of cervical T cells that are long-lived and elevated frequencies of cervical T cells in the intermediate of differentiation. Conversely, HIV infected women with reduced frequencies of CD4 T cells in their genital tracts are likely to have elevated frequencies of short-lived cervical TEff cells that are terminally differentiated.

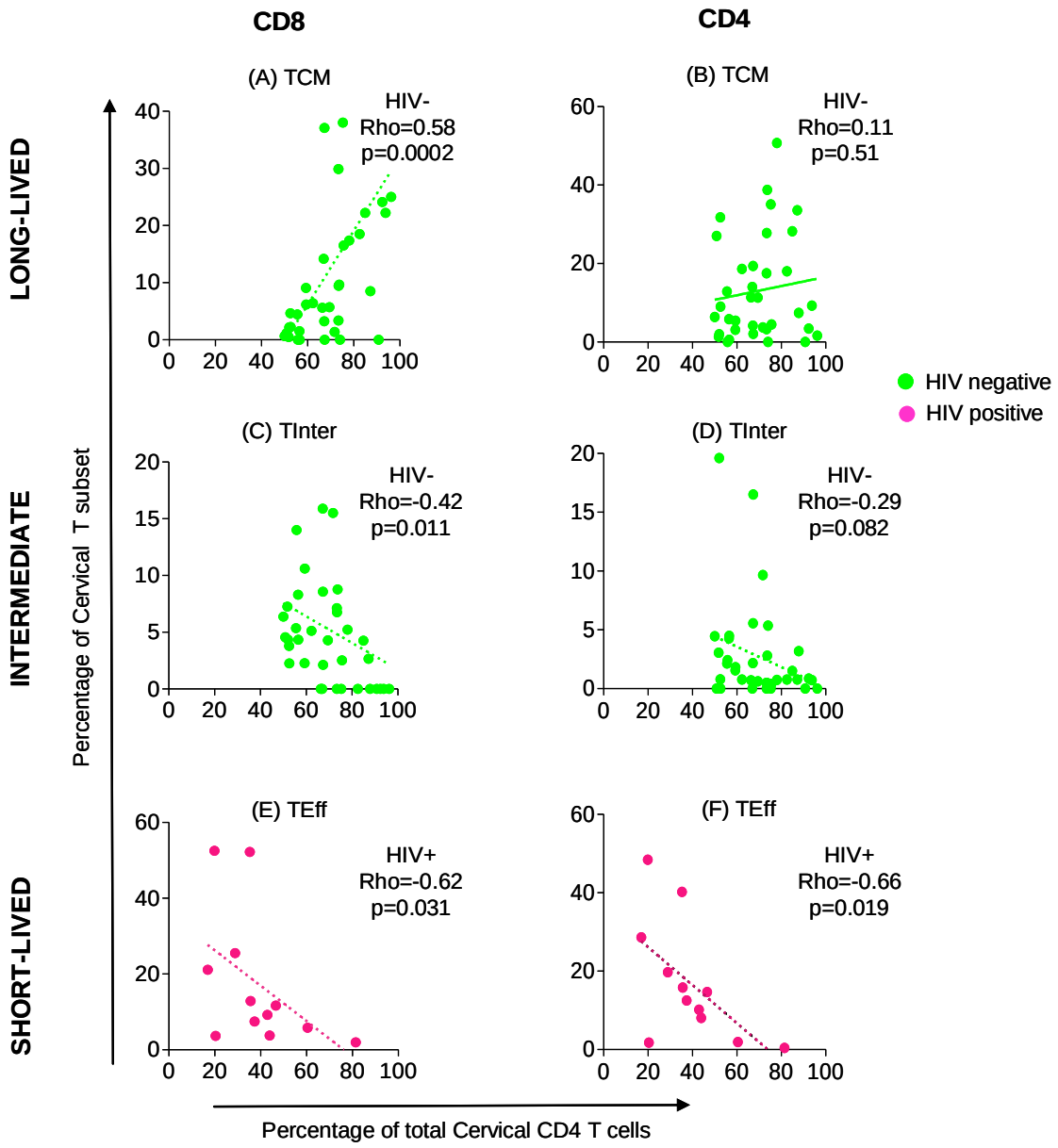

Figure 3.6 Relationship between cervical CD4 frequencies and distribution of CD8 (left panels) and CD4 (right panels) memory T subsets at the cervix. CD8 and CD4 memory subsets were separated into 3 groups of 'long-lived' [TCM (A) and (B)], 'intermediate' [(C) and (D) TInter], and 'short-lived' [TEff (E) and (F)] according to their stages of differentiation. Each dot represents an individual woman's proportions. Green dots represent HIV- women (n=36) and pink dots represent HIV+ women (n=12). Dotted lines represent best fits as predicted by linear regression. Spearman rank test was used to test the correlation. $P \leq 0.05$ were considered significant.

3.5 Discussion

This Chapter investigated the impact of HIV infection and HIV clinical status on the differentiation status of T cells derived from female genital tract. Like blood, I showed that the cervical mucosa of HIV-infected women had significantly reduced proportions of CD4 T cells than found in uninfected women. Consistent with the genital tract being a tissue effector site, T cells derived from the cervix were predominantly TEM in phenotype. .In HIV-infected women, HIV clinical status (indicated by CD4 counts in blood, and the frequency of CD4 T cells at the cervix) was associated with accumulation of T cells in the female genital tract in the late stages of differentiation. Furthermore, reduced proportions of CD4 T cells in the genital tract was associated with reduced frequencies of long-lived T cells and elevated frequencies of T cells in the intermediate and late stages of differentiation, that was independent of HIV infection status.

This Chapter shows that distribution of T memory subsets differ significantly between the female genital tract and blood. Since mucosal surfaces associated with the female genital tract are continually exposed to invading and colonizing microorganisms, maintenance of memory T cells at these exposed effector sites is important to provide the first line of defence against pathogenic invasion (Hayday *et al*., 2001; Nagler-Anderson *et al*., 2001; Masopust *et al*., 2006). The most dominant mucosal TEM cell pool may be regulated through interaction with local-site micro-organisms (MacPherson *et al*., 2004). After presentation of mucosal antigens by dendritic cells at the draining lymph nodes, the responding T cells traffic to the mucosal tissues where they reside as TEM cells (Jenkins *et al*., 2001). The constant presence of these TEM cells in the female genital tract with rapid effector functions (Sallusto *et al*., 1999; Yue *et al*., 2004; Romero *et al*., 2007) may provide protection at the cervical mucosa which is exposed to higher pathogen load than blood.

Conversely, TEM cells may be regulated through replenishment by TCM cells in order to maintain immune function at mucosal effector sites (Marzo *et al*., 2007; Okoye *et al*., 2007). Although TEM subsets were the dominant T cell subset found in HIV-infected and uninfected women in this study, TCM cells were found at the cervix and their frequency was elevated at the cervix compared to blood, significantly so for uninfected women. From this data, I hypothesize that local TCM subsets in the genital tract may serve to replenish TEM

cells in this compartment. This data also suggest that long-lived specific cellular immunity may be possible at this mucosal site, and this is encouraging for future mucosal vaccine studies. TCM have been identified at other mucosal sites in macaques such as in lungs, GALT and vagina (Poonia *et al*., 2006b; Marzo *et al*., 2007; Verhoeven *et al*., 2008). TCM cells that were identified in the mucosal effector sites in macaques have been proposed to be "reserve" antigen experienced cells that are in the "quasi-resting" state awaiting another exposure to the antigen of original stimulation (Poonia *et al*., 2006a).

TTM cells were also present in the female genital tract. Interestingly, these TTM and TCM CD4 T cells are the major cellular reservoirs for HIV (Chomont, *et al*., 2009) and their presence may ensure viral persistence in the female genital tract of HIV-infected women. The chronic presence of virus during HIV infection provides persistent antigen stimulation for T cells which is likely to influence their memory phenotype (Appay *et al*., 2002; Wherry *et al*., 2004). This study found that HIV-infected women had elevated frequencies of terminally-differentiated TEff at the cervix and in blood compared to uninfected women. Antigenic stimuli that are too strong or too prolonged, as is the case during chronic HIV infection, have been suggested to cause the expansion and differentiation of T cells to terminally-differentiated TEff cells that are no longer fit for development and survival (Wherry *et al*., 2004; Lanzavecchia and Sallusto, 2005). In this study, the impact of HIV infection on T cell differentiation was more pronounced in blood than at the cervical mucosa. This may be because the genital tract is marked by the accumulation of TEM cells which are known to preferentially migrate to tissue sites. The genital tract is also an inflammatory environment with comparatively high concentrations of cytokines such as IL-1β, IL-6, and IL-8 (Nkwanyana *et al*., 2009). The down-regulation of CCR7 and CD62L expression in TEM cells is induced by T cell activation and these changes alter the recirculation pattern of TEMs by allowing access to tissue sites of inflammation and decreasing recirculation through peripheral lymph nodes (Mackay *et al*.1991; 1992).

Reduced CD4 counts in blood and low proportions of CD4 T cells in the cervical compartment were significantly associated with higher frequencies of terminally-differentiated TEffs in the cervical compartment but not blood. Papagno *et al*. (2004) has previously described an inverse association between CD4 counts in blood during chronic HIV infection and the frequency of terminally-differentiated CD8 T cells in blood. Similarly,

Komanduri *et al*. (2007) showed that delayed immune reconstitution in HIV-infected women initiating anti-retroviral therapy was associated with skewing of the circulating T cell pool toward late memory cells. Although previous studies in acute and chronic HIV infection have shown a direct relationship between HIV viral load and T cell differentiation status (Ladell *et al*., 2008; Burgers *et al*., 2009), there was no association between the maturational status of T cells in both compartments and HIV viral load in this study. Burgers *et al*. (2009) also showed that the proportions of less differentiated TCM cells had a significant inverse correlation with activated CD8 cells as indicated by CD38 expression, whereas more differentiated TEM cells were found to have a significant positive association with activated CD8 cells. However, these studies were conducted in early HIV infection and not in chronically HIV-infected patients where activation of T cells is largely disorganized (Grossman *et al*., 2006). While the cause and effect relationship between HIV viral load, T cell differentiation profile and immune activation remains unclear, the findings of similarities in the majority of subsets between HIV-infected and uninfected women from this study suggest that differentiation of T cells is not driven directly by HIV but that immune activation in the presence of HIV may drive differentiation of T cells.

A significant positive correlation between absolute CD4 counts in blood and the frequency of CD8 TTM cells at the cervix was found. TTM cells have been reported to have initiated the remodelling events involved in acquisition of effector functions, however, depending on the level of stimulation these cells can be regenerated to TCM pool (Sallusto *et al*., 2004; Pearce and Shen, 2006). It was previously shown that CD4 restoration in the gut after HAART initiation was associated with an increase in both the TTM and TCM subsets (Macal *et al*., 2008; Verhoeven *et al*., 2008)..

Women with chronic HIV infection had significantly lower CD4 frequencies at the cervix than uninfected women, consistent with the findings from the studies done in rectal mucosa (Critchfield *et al*., 2008) and male genital tract (Politch *et al*., 2009). Elevated cervical CD4 frequencies were significantly associated with accumulation of long-lived T cells, whereas reduced cervical CD4 percentages were associated with accumulation of cervical T cells in the intermediate and late stages of differentiation. The presence of HIV itself or high level of immune activation may induce an environment that promotes increased rates of differentiation and loss of CD4 T cells.

Only 32% of the screened HIV-infected participants met inclusion criteria as opposed to 58% of the uninfected participants. Since the exclusion was based on cervical T cell factors (yield and function), it is possible that this exclusion processes introduced bias into the analysis of cervical T cell maturational status. Such bias could be minimized in future studies by using a viability marker to exclude dead cells as opposed to using another immunological factor such as function.

In conclusion, in this Chapter I describe the distribution of memory T cell phenotypes present in the cervical mucosa during HIV infection. The genital mucosa, like most other mucosal surfaces, is vulnerable to external pathogens and has been shown to have significantly elevated frequencies of TEM cells with limited proliferative potential that must rely on TCM cells present at this site for replenishment. The CD4 TCM cells, however, may serve as long-lived HIV reservoirs at this site. HIV-infected women have significantly increased levels of terminally-differentiated T cells compared to uninfected women. These terminally-differentiated T cells were found to be present in the highest numbers in women with lower CD4 counts in blood and lower proportions of cervical CD4 T cells. Reduced CD4 frequencies in the female genital tract was characterized by a reduction in T cells in the early stages of differentiation (TCM) and an increase in T cells in the intermediate and terminal stages of differentiation. Overall, these findings demonstrate an important link between HIV infection, clinical status and differentiation profile of T cells in the female genital tract.

Chapter 4

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Chapter 4
Defects in cytokine production associated with HIV-infection and T cell differentiation status in the female genital tract of HIV-infected women

4.1 Summary

Understanding the quality and quantity of HIV-specific immune responses in the female genital tract during HIV infection may help in the design of vaccine strategies aimed at containing HIV replication at the site of viral encounter. The aim of this Chapter was to investigate the impact of T cell maturational status and HIV-infection on the functional ability of T cells in the genital tract compared to blood. The maturational status of cervical cytobrush- and blood-derived T cells from 12 HIV-infected and 36 uninfected women was investigated by differential staining of T cells for CD45RA, CD27 and CCR7. Production of IFN-γ and IL-2 in response to stimulation with HIV Gag peptides and PMA/ionomycin by distinct memory T cell subsets in blood and cervix was compared. In response to PMA/ionomycin stimulation, significantly lower frequencies of genital tract CD4 TEM cells from HIV-infected women produced IFN-γ ($p=0.04$) and IL-2 ($p=0.01$) than uninfected women. In contrast, in uninfected women, significantly higher frequencies of genital tract CD4 TEM produced IFN-γ, IL-2 and dual IFN-γ/IL2 in response to PMA/ionomycin stimulation compared to CD4 TEM cells in blood ($p=0.03$; $p=0.002$; and $p=0.01$, respectively). This was not observed in HIV-infected women. No differences in the CD8 T cell compartment were noted. Few HIV-infected women had detectable IFN-γ and IL-2 HIV-specific T cell responses at the cervix and dual IFN-γ/IL2 HIV-specific responses were not present. This study identifies defects in both HIV-specific and global CD4 T cell memory cells in the female genital tract during chronic HIV infection.

4.2 Introduction

The differentiation status of HIV-specific T cell has been shown to play a significant role in the quality of HIV-specific responses and subsequent disease outcome (Addo *et al*., 2007; Northfield *et al*., 2007; Potter *et al*, 2007; Grevenynghe *et al*., 2008; Ladell *et al*., 2008; Burgers *et al*., 2009). Abnormal maturation of HIV-specific memory T cells has been implicated in the inability of the effector immune responses to clear HIV (Yue *et al*., 2004). The differentiation status of T cells has also been associated with both qualitative and quantitative differences in cytokine production (Sallusto *et al*., 1999; Masopust *et al*., 2001). TCM cells have been shown to produce mainly IL-2, whilst TEM cells have been shown to possess immediate effector functions (cytotoxicity and IFN-γ secretion) but reduced ability to proliferate and secrete IL-2 (Sallusto *et al*., 1999; Younes *et al*., 2003). Terminally-differentiated TEff cells, on the other hand, have been shown to have potent effector functions but limited proliferative capacity and fail to produce IL-2 (Halwani *et al*., 2006).

One of the characteristic immune defects associated with HIV infection is loss of HIV-specific CD4 T cells in blood that are able to secrete IL-2 (Younes *et al*., 2003). Instead of producing IL-2, Younes *et al*. (2003) found that HIV-specific CD4 T cell responses during chronic HIV infection were skewed towards producing IFN-γ only or were completely absent (Younes *et al*., 2003). Unfortunately, these HIV-specific CD4 T cells producing both IL-2 and IFN-γ have been shown to be associated with viral control (Harari *et al*., 2004) and non-progressive disease (Boazz *et al*., 2002; Iyasere *et al*., 2003; Harari *et al*., 2004; Emu *et al*., 2005). The persistence of HIV-specific IFN-γ/IL-2 secreting CD8 T cells were reported to promote CD8 T cell proliferation through secretion of IL-2 even in the absence of HIV-specific helper CD4 T cells (Zimmerli *et al*., 2005).

While there have been a number of studies investigating the role of CD8 and CD4 T cells in blood in controlling HIV-infection (Wilson *et al*., 2000; McNeil *et al*., 2001; Altfeld *et al*., 2006; Betts *et al*., 2006; Addo *et al*., 2007; Saez-Cirion *et al*., 2007), these HIV-specific T cells in blood ultimately fail to clear HIV and prevent transmission to uninfected sexual partners. There have been a number of studies showing that HIV-specific CD8 T cells are detectable in cervical mucosa of HIV-infected women (Musey *et al*., 1997; Kaul *et al*., 2003; Musey *et al*., 2003a; Gumbi *et al*., 2008; shown in Chapter 2). Few studies have provided

qualitative functional and maturational information on vaginal T cell responses to HIV and their role in transmission (Stevceva *et al*., 2002; Saba *et al*., 2010). In this study, I sought to compare the impact of T cell maturational status in the female genital tract and blood on polyclonal (PMA/ionomycin) and HIV Gag-specific CD8 and CD4 T cell responses (IFN-γ and IL-2) in HIV-infected and uninfected women. Information about the differentiation status and quality of immune cells responding to HIV at cervix may be useful in the development of mucosally effective HIV-1 vaccines.

4.3 Methods

4.3.1 Description of individuals included in the study

Thirty eight chronically HIV-infected and 62 uninfected women attending the Emphilisweni community clinic in Guguletu, Cape Town were enrolled in this study (also described in Chapter 3 Section 3.3.1). Women who were menstruating at the time of sampling, who were post-menopausal, had undergone a hysterectomy, had vaginal discharge, or visible or reported STI were excluded from the study. The study was approved by the Research Ethics Committee of the University of Cape Town, South Africa (UCT REC 258/2006) and informed written consent was obtained from all volunteers of the study.

4.3.2 Cervical sample collection and processing

Cervical cells were collected and isolated as previously described in Chapter 2 (Section 2.3.2).

4.3.3 PBMC isolation

PBMCs were isolated from ACD anti-coagulated whole blood as previously described in Chapter 2 (Section 2.3.5).

4.3.4 Counting of cervical and blood mononuclear cells

Cervical mononuclear cell numbers were evaluated by Guava automated cell counting (Guava technologies, Hayward, CA) as described previously in Chapter 2 (Section 2.3.4), and

adjusted to 0.1-1x10^6 cell/ml for *ex vivo* stimulation. PBMCs were counted as described previously in Chapter 2 (Section 2.3.6).

4.3.5 Intracellular cytokine staining and flow cytometry

Cervical and blood-derived T cells were evaluated for intracellular IFN-γ and IL-2 production in response to stimulation with HIV Gag peptides and PMA/ionomycin (positive control). Cervical cells (~0.1-1×10^6 cell/ml) and PBMC (1×10^6 cell/ml) were stimulated for 6 hours *ex vivo* at 37^{o}C and 5% CO_2 with (i) HIV subtype C (Du422) Gag peptides (122, 15-mer peptides overlapping by 10 amino acids; each peptide was present at a final concentration of 1 μg/ml, kindly provided by the NIH AIDS Reagent Repository), (ii) PMA/ionomycin (each at a concentration of 10 μg/ml, Sigma-Aldrich; positive control) or (iii) unstimulated (negative control). Brefaldin A (10 μg/ml, Sigma, St. Louis, MO) was added after the first hour. After 6 hours, stimulated cervical and blood-derived CD8 and CD4 T cells were stained for markers of T differentiation (CD45RA, CD27 and CCR7) and intracellular cytokines (IFN-γ and IL-2). Stimulated cells were initially washed 2× with 2 ml PBS (5 min, 300×g, 1300 rpm, room temperature). Cells were stained with pre-titrated anti-CD8-PerCP-Cy5.5 (BD Biosciences), anti-CCR7-APC (R&D Systems Inc., Minneapolis, MN), anti-CD27-PE (BD Biosciences) and anti-CD45RA-Cy7.PE (BD Biosciences) for 1 hour at 4ºC. Cells were washed with Perm/Wash buffer (2 ml; BD Biosciences) and permeabilized in Cytofix/CytoPerm (BD Biosciences) for 20 min at 4ºC. Cells were then stained with anti-CD3-pacific blue (BD Biosciences), anti-IFNγ-Alexa700 and anti-IL2-FITC for 1 hour at 4ºC. Cells were finally washed and fixed with Cell Fix (BD Biosciencies) and cell fluorescence was measured using a LSRII Flow Cytometer (BD Biosciences; San Jose, CA, USA). FlowJo software version 8.5.3 (Tree Star, Inc; Ashland, Oregon, OR, USA) was used for colour compensation and data analysis. Fluorescence minus one (FMO) and the unstimulated control were used to set gates.

4.3.6 Measurement of viral load in cervical supernatants and plasma

Viral load was determined in cervical supernatants and plasma samples using Nuclisens Easyq HIV 1 Version1.2 as previously described in Chapter 2 (Section 2.3.9). The detection limit of this assay was ≥50 copies/ml, and values less than 50 copies/ml were reported as zero.

4.3.7 Statistical analysis

Statistical analyses were performed using GraphPad Prism 5® (GraphPad Software, San Diego California USA). The Mann-Whiteny U and Students' unpaired T tests were applied for independent sample comparison. Spearman Ranks correlation was applied for assessing associations. P-values of ≤ 0.05 were considered significant.

4.4 Results

4.4.1 Clinical description of study participants

Of the 38 HIV-infected and 62 uninfected women enrolled, cervical samples from 12/38 (31.5%) of HIV-infected and 36/62 (58.1%) of uninfected women were suitable for analysis (described in detail in Chapter 3 section 3.4.1). The clinical details of 12 HIV-infected and 36 uninfected women that were analyzed have been previously summarized in Chapter 3 (Section 3.4.1, Table 3.1). Briefly, the median age of HIV-infected women was 35.0 years (IQR 28-43) versus 35.5 years for uninfected women (IQR 30-47). The cervical CD4:CD8 ratio was significantly less in HIV-infected women than uninfected women [median 0.58 (IQR 0.29-0.85) for HIV+ versus 2.17 (IQR 1.29-3.44) for HIV- women; $p<0.0001$]. All HIV-infected women were naïve to anti-retroviral therapy. HIV-infected women had a median CD4 T cell count of 292 cell/µl (IQR 142-559). Their median HIV viral load was 3000 RNA copies/ml in plasma (IQR 180-31000) and 200 RNA copies/ml in cervical secretions (IQR 0-540).

4.4.2Characterization of cytokine production profiles of CD8 and CD4 T cells in the genital tract and blood following stimulation with PMA/ionomycin

To investigate the impact of HIV infection on "global" cytokine production by T cells from the cervix and blood, the ability of T cells to produce IFN-γ and IL-2 following stimulation with PMA/Ionomycin was compared in HIV-infected and uninfected women (Figure 4.1). The median frequencies of cervical CD8 and CD4 T cells producing IFN-γ and IL-2 spontaneously (background) was generally undetectable and there was no significant difference between background frequencies of IFN-γ+, IL-2+ or IFN-γ+IL-2+ (referred to as dual positive hereafter) cervical T cells derived from HIV-infected and uninfected women (data not shown). In both cervical and blood compartments, CD8 T cells secreted mainly

IFN-γ upon polyclonal stimulation irrespective of HIV status (Figure 4.1A and B). No significant differences were noted in the ability of genital tract CD8 T cells from HIV-infected and uninfected women to secrete IFN-γ and IL-2 either alone or in combination (Figure 4.1B). Similarly in blood, there was no significant difference in the ability of peripheral blood CD8 T cells from HIV-infected and uninfected women to secrete IFN-γ and IL-2 either alone or in combination (Figure 4.1B). In HIV uninfected women, significantly higher frequencies of genital tract CD8 T cells produced IFN-γ compared to peripheral blood CD8 T cells (p=0.01; Figure 4.1B).

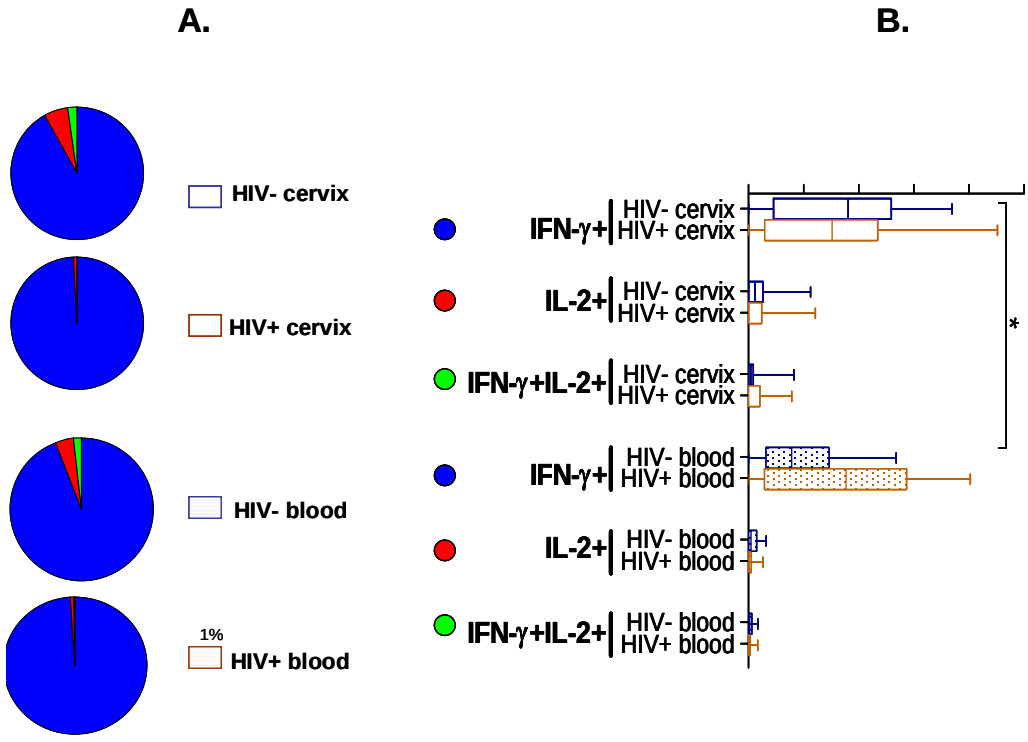


Figure 4.1 Comparison of the ability of CD8 T cells from cervix and blood of HIV-infected and uninfected women to produce IFN-γ and IL-2 after global stimulation with PMA/ionomycin. A. Pie charts represent the proportion of CD8+ T cells producing IFN-γ alone (blue), IL-2 alone (red) or both IFN-γ and IL-2 (green) in response to PMA/ionomycin stimulation. **B**. Comparison of the net frequencies CD8 T cells from the cervix (top panels) and blood (bottom panels) from HIV negative (clear boxes) and HIV positive (filled boxes) women to produce IFN-γ and IL-2 following stimulation with PMA/ionomycin. Responses were compared for each group using the Mann-Whitney U test and $p \leq 0.05$ were considered significant (*$p<0.05$).

In contrast, CD4 T cells were found to secrete both IFN-γ and IL-2 (Figure 4.2A) upon polyclonal stimulation. HIV-infected women had 3-fold lower IFN-γ and IL-2 responses at

the cervix than uninfected women although this was not significant (Figure 4.2 A and B). Genital tract CD4 T cells from uninfected women had significantly higher frequencies of IFN-γ+ (p=0.02; median of 13.0% at the cervix compared to 9.5% in blood), IL-2+ (p=0.01; median of 13.1% at the cervix compared to 7.43% in blood) and dual cytokine+ (p=0.005; median of 3.9% at the cervix compared to 1.8% in blood) responses compared to blood CD4 T cells (Figure 4.2B). In HIV-infected women, however, no such differences were noted in the ability of cervical versus blood CD4 T cells to produce IFN-γ (p=0.47; median of 4.6% at the cervix compared to 10.3% in blood), IL-2 (p=0.58; median of 3.8% at the cervix compared to 3.6% in blood) and dual cytokines (p=0.93; median of 1.1% in blood compared to 0.9% in blood). The lack of statistical significance could be due to a lower number of HIV-infected participants.

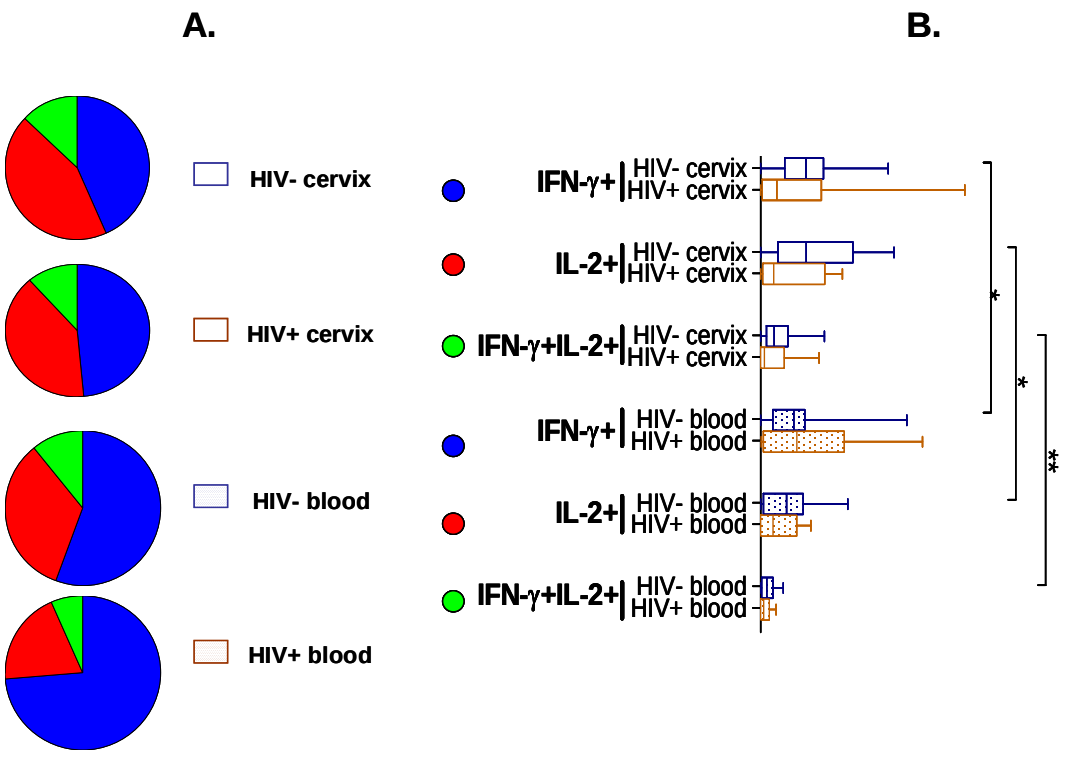


Figure 4.2 Comparison of the ability of CD4 T cells from cervix and blood of HIV-infected and uninfected women to produce IFN-γ and IL-2 after global stimulation with PMA/ionomycin. A. Pie charts represent the median frequency of positive responses and show the proportion of cells positive for IFN-γ alone (blue), IL-2 alone (red) or dual positive (green). **B**. Comparison of the net frequencies of CD4 T cells from the cervix (top panels) and blood (bottom panels) of HIV negative and HIV positive women to produce IFN-γ and IL-2 following stimulation with PMA/ionomycin. Responses were compared for each group using the Mann-Whitney U test and p≤ 0.05 were considered significant (*p<0.05; and **p<0.005).

4.4.3 The impact of T cell maturational status on the global cytokine production profiles of T cells in the genital tract and blood

To examine the influence of T cell differentiation status on the functional ability of T cells from HIV-infected and uninfected women, IFN-γ and IL-2 production following polyclonal stimulation with PMA/ionomycin by distinct T cell memory subsets was investigated. The 6 different T subsets were identified using surface phenotypic markers CD45RA, CCR7 and CD27 previously described in Chapter 3 (Table 3.2) and stratified according to their survival capability (long-lived to short-lived): Tn (CD45RA+CCR7+CD27+)→ TCM (CD45RA-CCR7+CD27+)→ TTM (CD45RA-CCR7-CD27+)→ TEM (CD45RA-CCR7-CD27-)→ TInter (CD45RA+CCR7-CD27+)→ TEff (CD45RA+CCR7-CD27-; Sauce *et al.*, 2007). Surface phenotypes of antigen-specific cells were defined by overlaying antigen-specific T cells on a background of total CD8+ or CD4+T cells and examining expression of CD45RA, CCR7 and CD27. Figure 4.3 shows the gating strategy used to define distinct memory subsets of functional T cells. Activation of T cells with PMA/ionomycin has the advantage of activating sufficient proportions of rare T cells to give information about cytokine signature of each subset (Kim *et al.*, 2009).

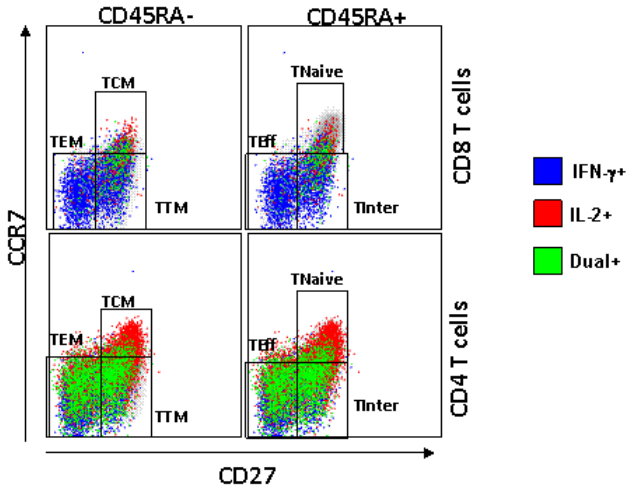


Figure 4.3 Representative plots showing the gating strategy used to identify the memory phenotype of T cells responding to PMA/ionomycin. Density and overlay plots showing frequencies of IFN-γ (blue dots), IL-2 (red dots) and dual cytokine (green dots) secreting CD8 (upper panels) and CD4 T cell subsets (lower panels) expressing CD27, CCR7 or CD45RA. FMO controls were used to set gates.

The impact of tissue location, HIV status and memory T cell status on the global cytokine production capacity of CD8 T cells was investigated (Figure 4.4). CD8 memory T subsets

predominantly produce IFN-γ with reduced ability to secrete IL-2. Overall, the order of IFN-γ producing CD8 T subsets in blood and cervix of all women was as follows: TCM<TInter<TEff<TEM<TTM cells (from the least responsive on the left, to the most responsive on the right). Conversely, for IL-2 producing CD8 T subsets, the order was as follows: TEff<TInter<TEM<TCM<TTM (from the least responsive on the left, to the most responsive on the right).

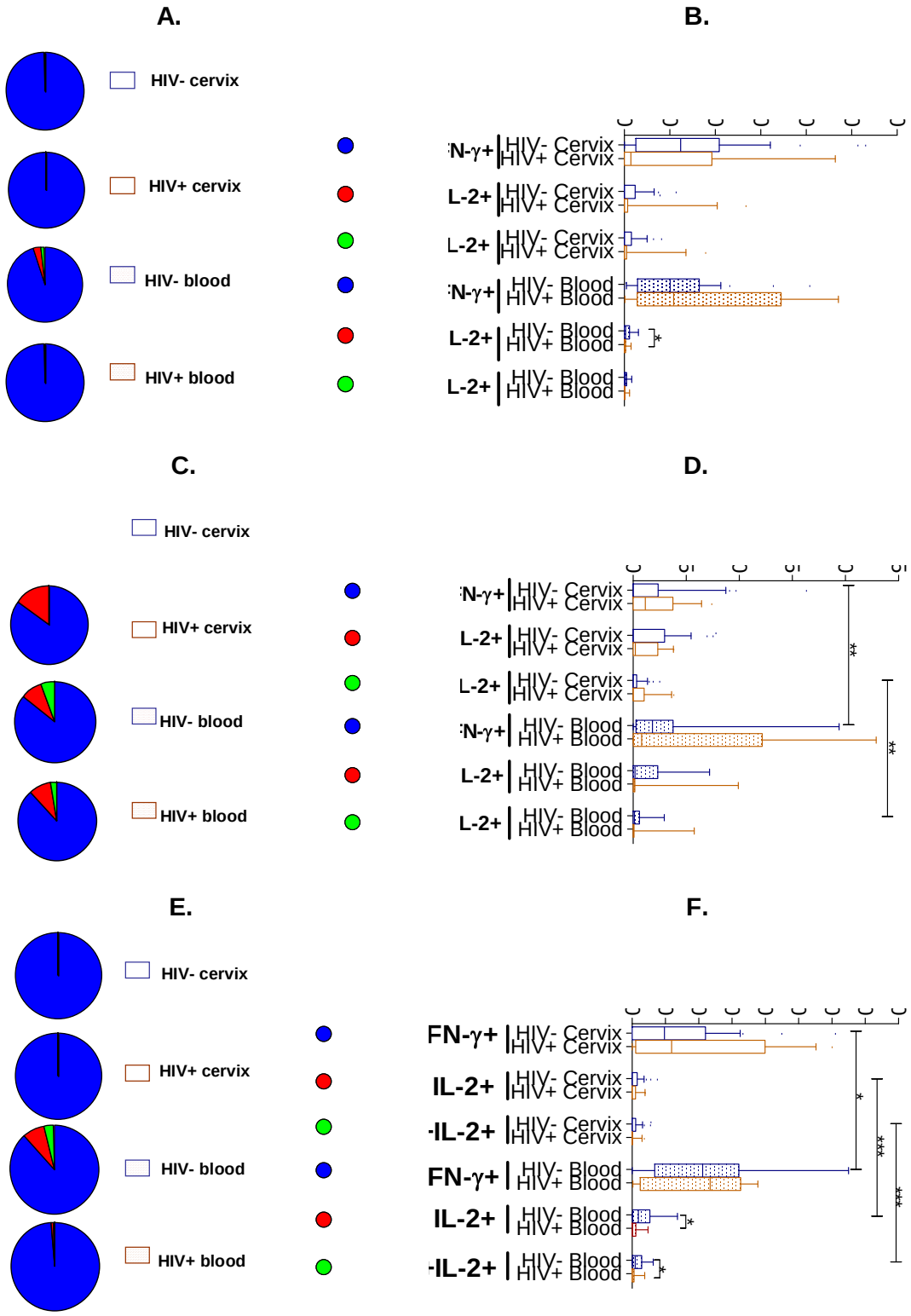
4.4.3.1 Comparison of PMA/ionomycin responses by memory CD8 T cells in HIV-infected versus uninfected women

In the cervical compartment, it was found that the frequency of IFN-γ, IL-2 and dual IFN-γ/IL-2 responses to PMA/ionomycin stimulation by all cervical CD8 T memory subsets was similar in HIV-infected versus uninfected women. Similarly in blood, the frequency of IFN-γ responses by CD8 T memory subsets was comparable in HIV-infected versus uninfected women. However, the frequency of IL-2 responses was significantly higher in blood of HIV negative women compared to HIV positive women in TEM ($p=0.04$) and TTM ($p=0.02$) subsets (Figure 4.4).

4.4.3.2 Comparison of PMA/ionomycin responses by memory CD8 T cells in blood versus cervical compartments

In HIV infected women, cervical versus blood CD8 T memory subsets had similar frequencies of IFN-γ, IL-2 and dual IFN-γ/IL-2 responses (Figure 4.4). In HIV-uninfected women, however, the majority of CD8 T cell subsets (TCM, TTM and TInter) in blood had significantly higher functional ability compared to those found in the cervical compartment. There were significantly higher frequencies of blood TCM cells that produced IFN-γ and dual IFN-γ+IL-2+ compared to cervical TCM cells in uninfected women ($p=0.008$ and $p=0.005$, respectively; Figure 4.4D). Similarly, significantly higher frequencies of TTM cells in the blood compartment produced IFN-γ+, IL-2+ and dual IFN-γ+IL-2+ responses compared to cervical TTM cells ($p=0.03$; $p<0.0001$ and $p=0.0004$ respectively; Figure 4.4F). Finally, significantly higher frequencies of CD8 TInter cells in blood from uninfected women produced IL-2+ and dual IFN-γ+IL-2+ compared to TInter cells at the cervix ($p=0.0002$ and $p<0.0001$, respectively; Figure 4.4H).

In summary, although CD8 TEM cells are the most dominant T cell subset found in the cervix and were detected at significantly higher frequencies compared to blood, the magnitude of IFN-γ+, IL-2+ and dual IFN-γ+IL-2+ CD8 TEM cells were similar in blood versus cervix, irrespective of HIV status. In the absence of HIV infection, the majority of CD8 T cell subsets (TCM, TTM and TInter) in blood had significantly higher functional ability compared to those found in the cervical compartment. However, in HIV-infected women, cytokine production potential by CD8 T subsets was not impacted by tissue location. Lack of IL-2 production during chronic HIV infection was only observed in blood CD8 TEM and TTM cells.


A.
HIV- cervix
HIV+ cervix
HIV- blood
HIV+ blood
B.
N-γ+
L-2+
L-2+
N-γ+
L-2+
L-2+
HIV- Cervix
HIV+ Cervix
HIV- Blood
HIV+ Blood
*
C.
HIV- cervix
HIV+ cervix
HIV- blood
HIV+ blood
D.
N-γ+
L-2+
L-2+
N-γ+
L-2+
L-2+
**
**
E.
HIV- cervix
HIV+ cervix
HIV- blood
HIV+ blood
F.
FN-γ+
IL-2+
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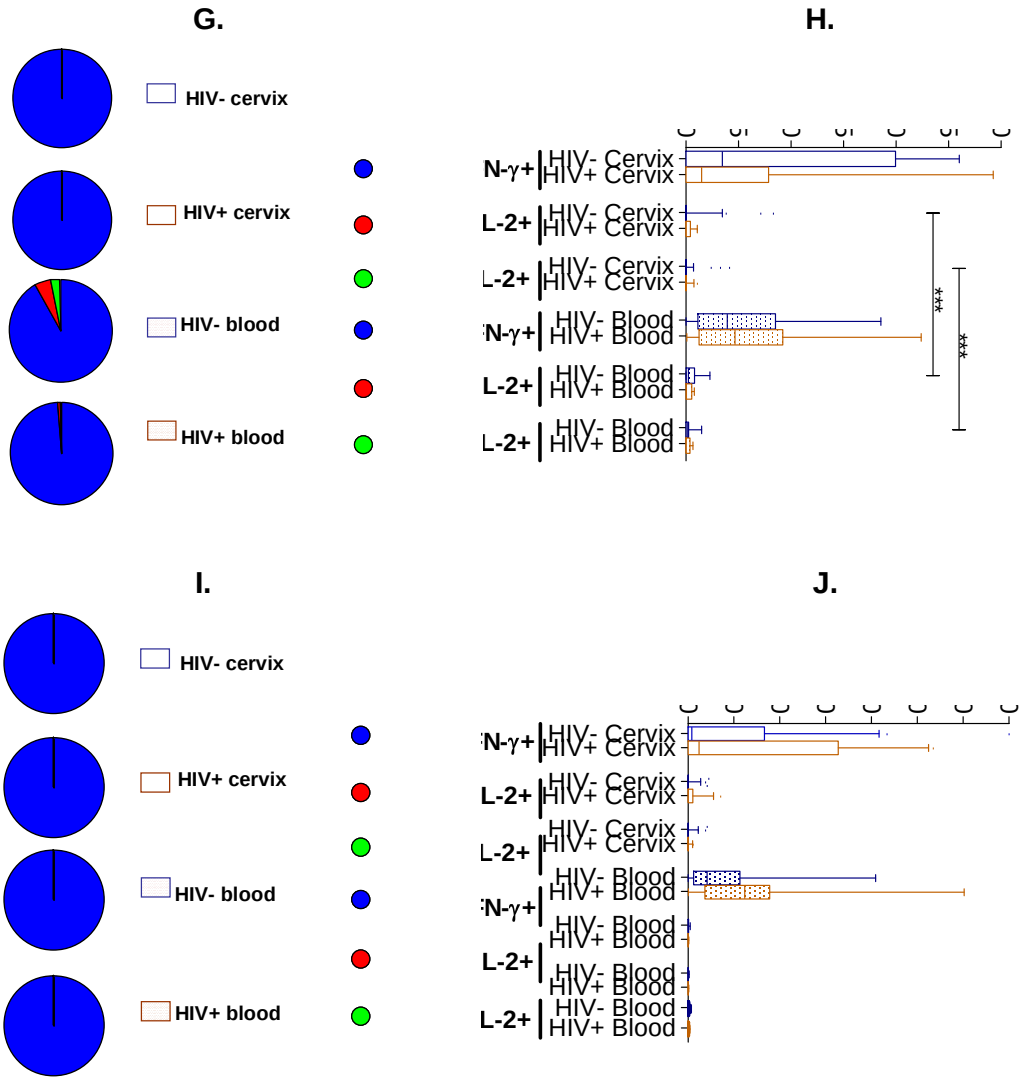


Figure 4.4 Comparison of the ability of CD8 T subsets from cervix and blood of HIV-infected and uninfected women to produce IFN-γ and IL-2 after global stimulation with PMA/ionomycin. A, C, E, G and I show pie charts representing the median frequency of positive responses and show the proportion of memory subsets positive for IFN-γ alone (blue), IL-2 alone (red) or dual positive (green). B,D, F, H and J show comparison of the net frequencies CD8 T memory subsets from the cervix (top panels) and blood (bottom panels) in HIV negative and HIV positive women to produce IFN-γ, IL-2 or both cytokines following stimulation with PMA/ionomycin. Magnitude of responses by (B) TEM, (D) TCM, (F) TTM, (H) TInter and (J) TEff subsets (based on expression of phenotypic markers CD45RA, CCR7 and CD27) were compared for each group using the Mann-Whitney U test and $p \leq 0.05$ were considered significant (*$p<0.05$; **$p<0.005$ and ***$p<0.001$).

The functional ability of CD4 T cells were compared in blood versus cervix or in HIV-infected versus uninfected women (Figure 4.5). Not only are the CD4 T cells important in the development of CD8 T cell responses (Shedlock and Shen, 2003) by virtue of their helper function, they have also been shown to play a role in the control of HIV load (Rosenberg *et al*., 2000; Boritz *et al*., 2004; Harari *et al*., 2004). CD4 T subsets secreted both IFN-γ and IL-2 cytokines. Overall, the order of IFN-γ producing CD4 T subsets in blood and cervix of all women was as follows: TCM<TInter<TEff<TTM<TEM cells (from the least responsive on the left, to the most responsive on the right). Conversely, for IL-2 producing CD4 T subsets, the order was as follows: TEff<TInter<TCM<TEM<TTM (from the least responsive on the left, to the most responsive on the right).

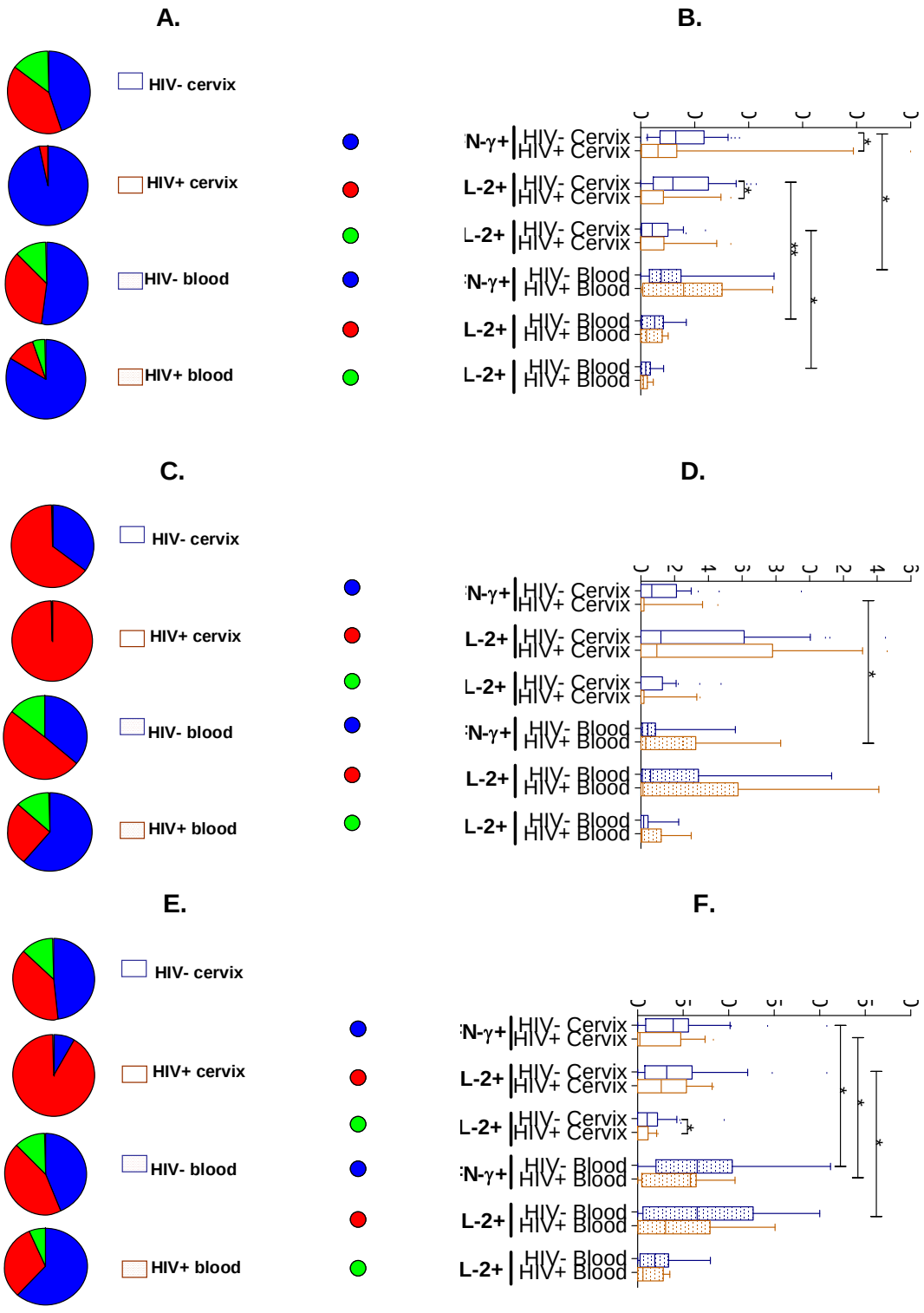
4.4.3.3 Comparison of PMA/ionomycin responses by memory CD4 T cells in HIV-infected versus uninfected women

In the cervical compartment, it was found that the frequency of IFN-γ, IL-2 and dual IFN-γ/IL-2 responses to PMA/ionomycin stimulation by majority of cervical CD4 T memory subsets was similar in HIV-infected versus uninfected women. Interestingly, the frequencies of both IFN-γ and IL-2 responses by CD4 TEM cells, which is the major CD4 subset in the cervical compartment, was significantly higher at the cervix of HIV negative women compared to HIV positive women ($p=0.04$ and $p=0.01$, respectively; Figure 4.5B). Since cervical CD4 TEM cells are found at similar frequencies in both HIV-infected and uninfected women (Chapter 3 Section 3.4.4), these results suggest that lack of both IL-2 and IFN-γ production by cervical TEM cells in HIV-infected women is due to dysfunction of CD4 TEM cells rather than their depletion. The frequency of dual IFN-γ and IL-2 cytokine production by CD4 TTM cells was also significantly less in HIV-infected women compared to those from uninfected women ($p=0.04$; Figure 4.5F). When similar comparisons were made in blood compartment, IFN-γ+, IL-2+ and dual IFN-γ+IL-2+ responses by all CD4 T memory subsets were similar in HIV-infected versus uninfected women.

4.4.3.4 Comparison of PMA/ionomycin responses by memory CD4 T cells in blood versus cervical compartments

Contrary to CD8 memory T subsets, responses by CD4 memory T subsets were impacted significantly by tissue location. In HIV infected women, almost all blood CD4 T memory subsets (TCM, TTM, Tinter and TEff) with the exception of TEM cells, had significantly higher frequencies of IFN-γ responses compared to cervical CD4 memory subsets (TCM p=0.05, TTM p=0.03, TInter p=0.004 and TEff p=0.004 Figure 4.4). In HIV-uninfected women, the majority of CD4 T cell subsets (TTM, TInter and TEff) in blood had significantly higher functional ability compared to those found in the cervical compartment. The frequency of CD4 TTM cells producing IFN-γ and IL-2 was significantly higher in blood compared to cervix (p=0.03 and p=0.04, respectively Figure 4.5F). TInter and TEff cells are a minor population in the cervical compartment. In line with them being only a minor population at the cervix, the frequencies of CD4 TInter and TEff cells in the female genital tract producing IFN-γ (TInter p=0.0004, TEff p<0.0001), IL-2 (TInter p<0.0001, TEff p=0.0004) and dual IFN-γ and IL-2 cytokines (TInter p<0.0001, TEff p<0.0001) were reduced compared to blood (Figure 4.5H and J). CD4 TEM subset in HIV-uninfected was different from the other subsets in that it was the frequency of cervical IFN-γ, IL-2 and dual IFN-γ+IL2+ responses that were significantly higher compared to TEM cells in blood (p=0.03; p=0.002; and p=0.01, respectively; Figure4.5A and B).

In summary, superior cytokine producing ability by mucosal CD4 TEM cells in the absence of HIV infection was observed. Significantly higher frequencies of CD4 TEM cells in the female genital tract of HIV uninfected women produced IFN-γ, IL-2 and dual IFN-γ+IL2+ compared to blood. In HIV-infected women, however, responses by CD4 TEM cells were similar at the cervix and in blood. Furthermore, cervical CD4 TEM cells in HIV-infected women had significantly reduced ability to produce IFN-γ and IL-2 upon polyclonal stimulation. Overall, these findings suggest a defect of CD4 T-cell-dependent immunity in the cervical TEM cells of HIV infected women.


A.
HIV- cervix
HIV+ cervix
HIV- blood
HIV+ blood
B.
N-γ+
L-2+
L-2+
N-γ+
L-2+
L-2+
HIV- Cervix
HIV+ Cervix
HIV- Blood
HIV+ Blood
C.
HIV- cervix
HIV+ cervix
HIV- blood
HIV+ blood
D.
E.
HIV- cervix
HIV+ cervix
HIV- blood
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F.

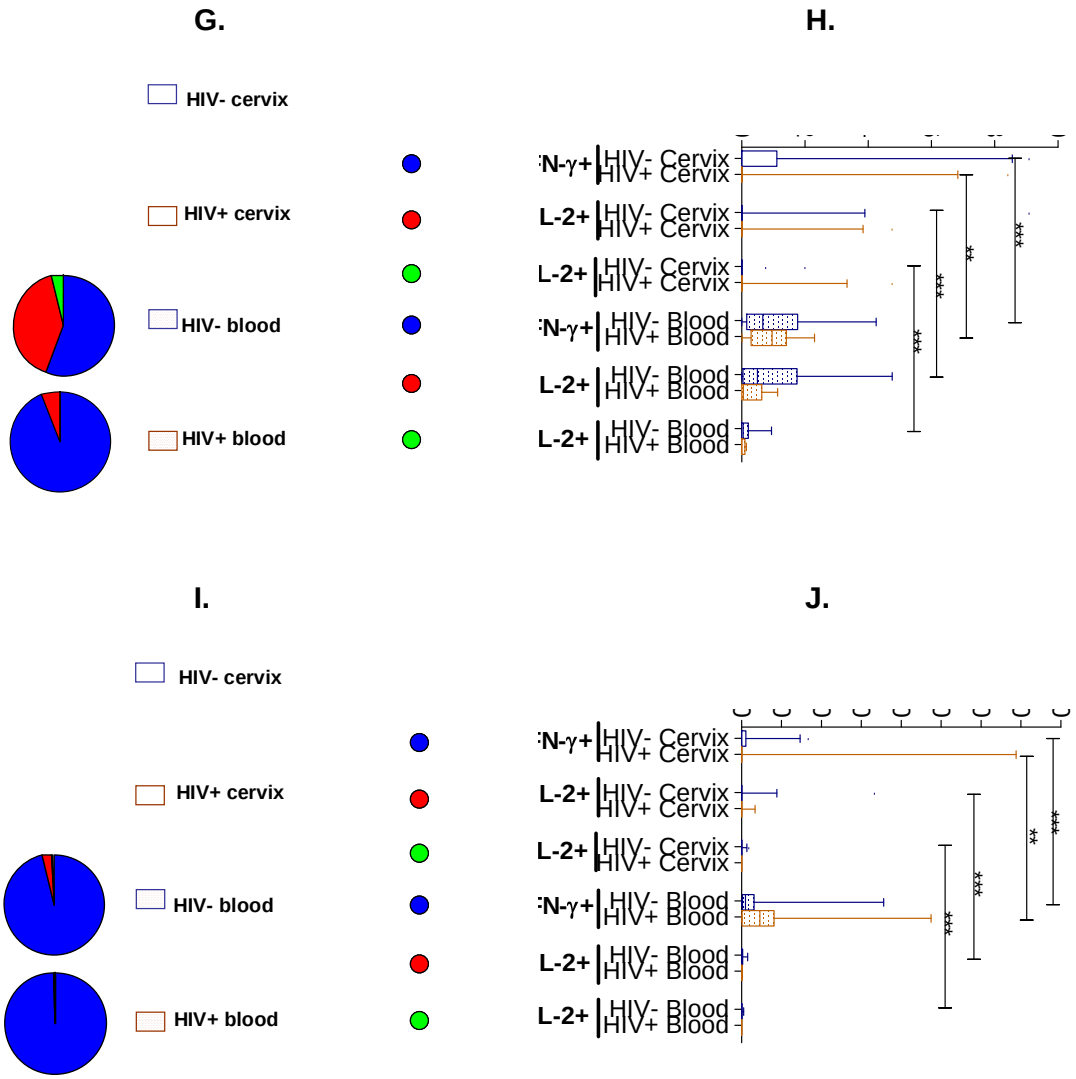

Figure 4.5 Comparison of the ability of CD4 T subsets from cervix and blood of HIV-infected and uninfected women to produce IFN-γ and IL-2 after global stimulation with PMA/ionomycin. A, C, E, G and I show pie charts representing the median frequency of positive responses and show the proportion of memory subsets positive for IFN-γ alone (blue), IL-2 alone (red) or dual positive (green). B,D, F, H and J show comparison of the net frequencies CD4 T memory subsets from the cervix (top panels, dark blue boxes) and blood (bottom panels, brown boxes) in HIV negative and HIV positive women to produce IFN-γ and IL-2 following stimulation with PMA/ionomycin. Magnitude of responses by (B)TEM, (D)TCM, (F)TTM, (H)TInter and (J)TEff subsets (based on expression of phenotypic markers CD45RA, CCR7 and CD27) were compared for each group using the Mann-Whitney U test and $p \leq 0.05$ were considered significant (*$p<0.05$; **$p<0.005$ and ***$p<0.001$).

4.4.4 HIV-1 Gag-specific T cell responses at the cervix and blood in HIV-infected women

The differentiation status of HIV Gag-specific T cells in blood and cervix in HIV-infected women was determined. Following stimulation with HIV Gag peptides, the magnitude and frequency of IFN-γ responses by total CD8 T cells from the cervix were significantly lower than those detected in blood in HIV-infected women ($p=0.0015$, Figure 4.6A) with only 1/12 HIV-infected women having clearly detectable Gag-specific CD8 T cell responses at the cervix. There was no significant difference in the overall magnitude of CD8 HIV-specific IL-2 responses between the two compartments although more women had Gag-specific IL-2 responses by CD8 T cells at the cervix than in blood. The frequency of IFN-γ responses to HIV gag peptides by CD4 T cells in both blood and cervical compartments were similar and lower than that of CD8 T cells (Figure 4.6B).

In response to HIV Gag, the frequency of blood CD8 and CD4 T cells producing IFN-γ alone were significantly higher than those producing IL-2 alone ($p=0.002$ for CD8 and $p=0.002$ for CD4 T cells) suggesting a skewing towards a dominant IFN-γ profile in blood during chronic HIV infection (similar to the findings of Younes *et al*., 2003 and Harari *et al*., 2004). This was not observed in the cervical compartment where Gag-specific responses were largely absent. In addition, T cells producing both IFN-γ and IL-2 simultaneously were not present in either anatomical compartment (Figure 4.6).

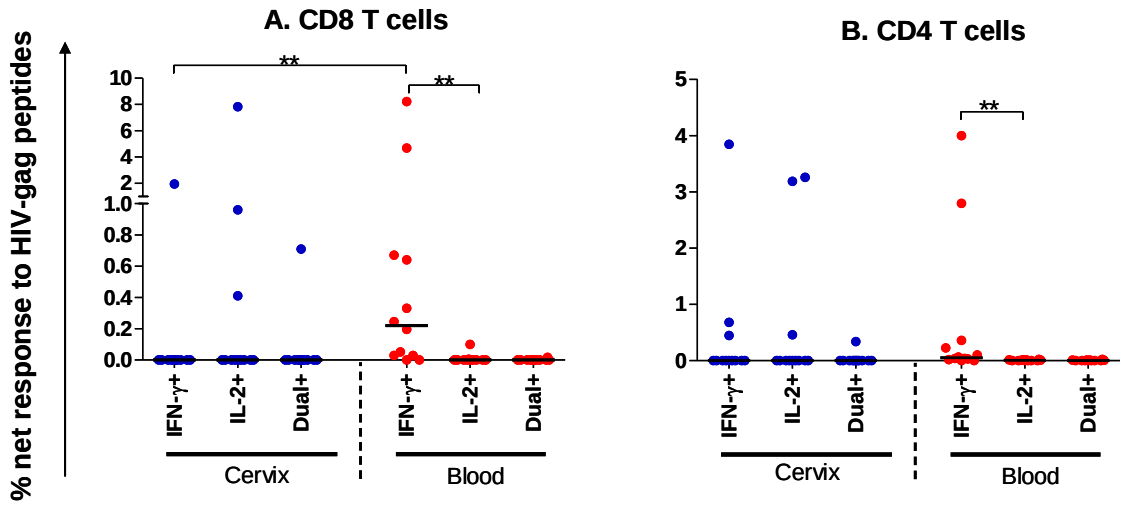

Figure 4.6 Characterization of cytokine production profiles of HIV Gag-specific CD8 (A) and CD4 (B) T cell responses during chronic HIV infection. **A.** Comparison of HIV-specific responses in cervical and blood CD8 T cells. **B**. Comparison of HIV-specific responses in cervical and blood CD4 T cells. The frequency of IFN-γ, IL-2 and dual cytokine secretion by T cell subsets in each of the two compartments was analyzed. Each dot represents each individual and horizontal lines represent median responses. The frequencies of responses were compared for each group using the Mann-Whitney U test. Asterisks indicate significant differences between groups (**$p<0.005$). Net responses were calculated by subtracting unstimulated cytokine responses from responses measured following Gag stimulation.

The differentiation status of T cells in blood and at the cervix responding to HIV Gag was determined in HIV-infected women (Figure 4.7 and Figure 4.8). In blood, Gag-responsive CD8 T cells were found to belong to TEM, TEff, TTM, and TInter memory subsets. In contrast, cervical CD8 T cells from only 1/12 HIV-infected women (8.3%) produced IFN-γ in response to stimulation with HIV Gag peptides and the responding CD8 T cells were found to be TEM (Figure 4.7A top panel). Interestingly, this woman did not have a matching IFN-γ response in blood to Gag confirming the lack of an association between cervical and blood compartments (as previously shown in Chapter 2).

IL-2+ responses to HIV Gag by CD8 T cells were detectable at cervix in 2/12 women (16.7%) and IL-2 was secreted predominantly by CD8 TCM cells (Figure 4.7B top panel). None of HIV-infected women had IL-2 responses by CD8 T cells to HIV Gag in the blood compartment (Figure 4.7B bottom panel).

In the CD4 T cell compartment, only 1/12 (8.3%) of the women studied (donor 7) had a clearly detectable CD4 IFN-γ response at the cervix in response to HIV Gag stimulation

(Figure 4.8A upper panel) and this was the same women who had a detectable CD8 T cell response (Figure 4.7A upper panel). In this woman, Gag-specific IFN-γ production was mediated by terminally-differentiated TEff CD4 T cells. In blood, 2/12 women had high magnitude IFN-γ responses to Gag which were dominated by TEff and TEM cells (Figure 4.8A bottom panel).

More HIV-infected women had HIV-specific CD4 T cells producing IL-2 at the cervix than IFN-γ although this was not significant. Three of the 12 HIV-infected women (donors 1, 10 and 12) had >3% of CD4 T cells at the cervix producing IL-2 in response to Gag, and these CD4 T cells were TEM and TTM cells in two donors (1 and 10) and TTM, TInter and TEff cells in donor 12 (Figure 4.8B top panel). In blood, none of the 12 HIV-infected women evaluated had IL-2 Gag-specific CD4 T cells (Figure 4.8B bottom panel). There was no correlation between CD4 T cells responses detected at the cervix and in blood.

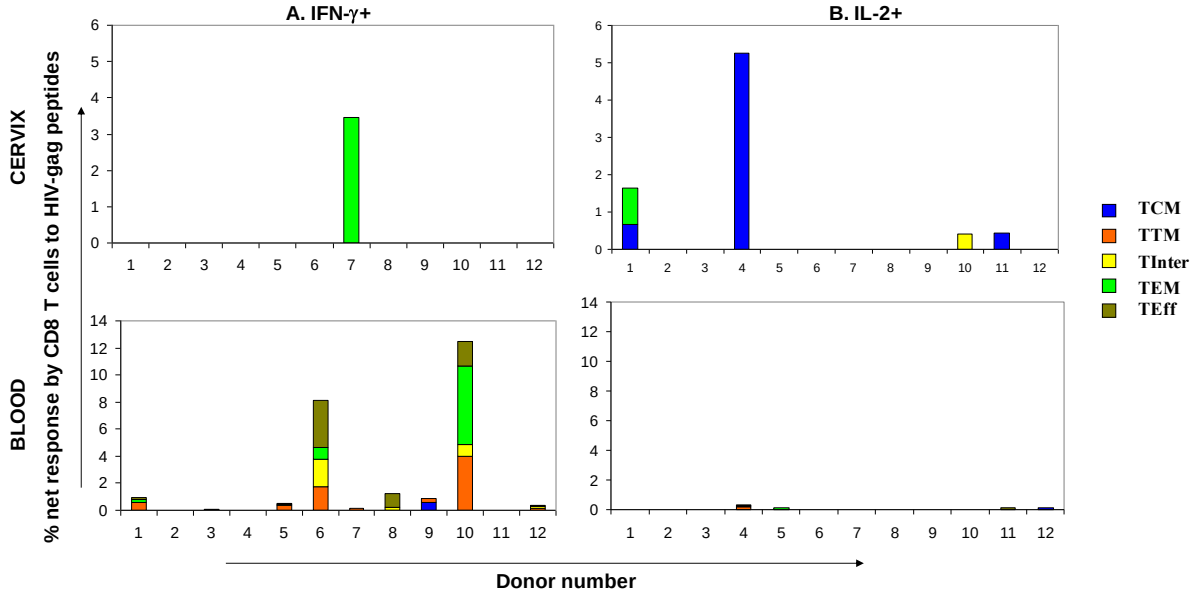

Figure 4.7 Frequency of HIV Gag-specific responses by distinct memory CD8 T cell subsets from the cervix and blood of HIV-infected women. The frequency of Gag-specific T cell IFN-γ (A) and IL-2 (B) responses by CD8 TCM (blue bars), TTM (red bars), TInter (yellow bars), TEM (green bars) and TEff (olive bars) derived from the cervix and blood were identified. All frequencies were adjusted for background by subtracting unstimulated cytokine production from Gag-specific responses by each T cell subset.

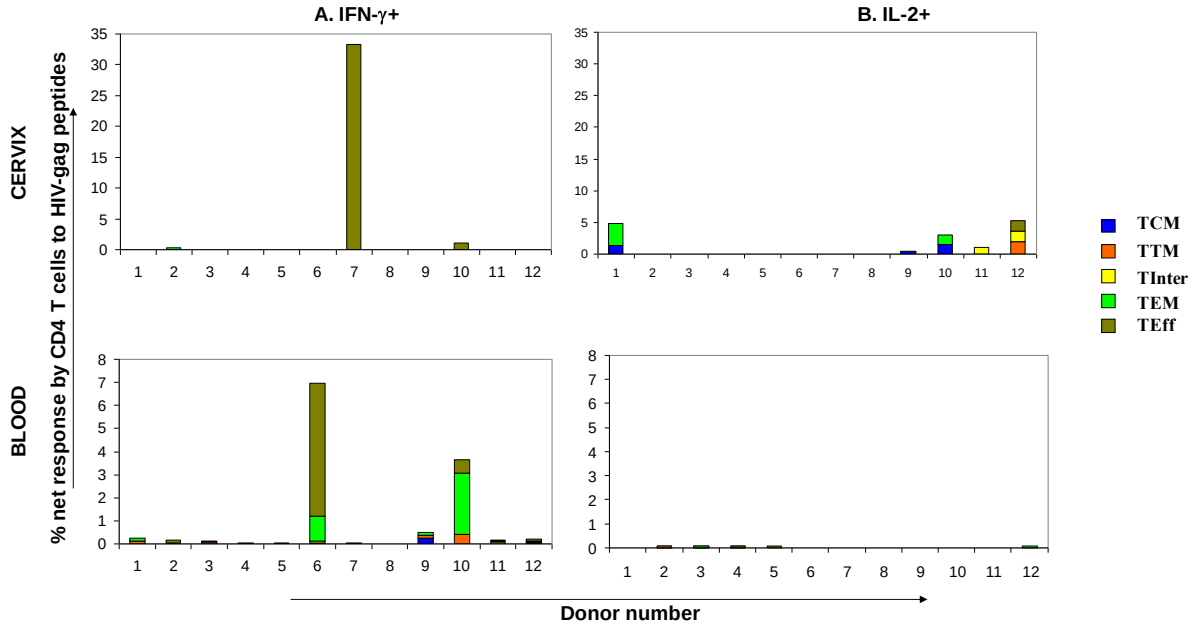

Figure 4.8 Frequency of HIV Gag-specific responses by distinct memory CD4 T cell subsets from the cervix and blood of HIV-infected women. The frequency of Gag-specific T cell IFN-γ (A) and IL-2 (B) responses by CD4 TCM (blue bars), TTM (red bars), TInter (yellow bars), TEM (green bars) and TEff (olive bars) derived from the cervix and blood were identified. All frequencies were adjusted for background by subtracting unstimulated cytokine production from Gag-specific responses by each T cell subset.

4.5 Discussion

Recent studies have shown that the quality rather than the quantity of HIV-specific T cell responses is important in control of HIV infection (Boaz *et al*., 2002; Betts *et al*., 2006; Harari *et al*., 2006; Day *et al*., 2007; Potter *et al*., 2007). Furthermore, the maturational status of HIV-specific T cells has also been suggested to influence quality of effective HIV-specific T cell responses (Addo *et al*., 2007; Northfield *et al*., 2007; Burgers *et al*., 2009). This Chapter describes the functional and phenotypic properties of global (PMA/ionomycin) and HIV Gag-specific T cell responses by CD8 and CD4 T cell memory subsets in the genital tract during chronic HIV-infection. A significant reduction in the frequency of CD4 TEM cells in the cervix producing IFN-γ and IL-2 in response to PMA/ionomycin stimulation during HIV infection was observed. This significant decrease in the frequency of responses is not as a result of lower overall CD4 TEM cell frequencies in HIV-infected women (Chapter 3). The results presented in this Chapter demonstrate mucosal CD4 T cell subset imbalances associated with HIV infection.

Homann *et al*. (2001) suggested that activated CD8 T cells are likely to expand in response to HIV infection, while similarly activated CD4 T cells are deleted. The net result of this being stable CD8 T cells numbers but a declining CD4 memory pool (Homann *et al*., 2001). Since cervical CD8 TEM cell responses were similar at the cervix and blood and not impacted by HIV status, this suggests that CD8 T cells may be less likely to be impacted by their tissue location and that they retain the ability to proliferate upon antigenic stimulation even in HIV-infected individuals.

CD4 TEM cells expressing CCR5 have previously been identified as the major memory T cell subset at mucosal effector sites and these cells are selectively infected and depleted during SIV infection (Veazey *et al*., 1998; 2000a; 2000b; 2003; Groot *et al*., 2006). Many studies have shown that mucosal TEM cells play a critical role in mucosal protection against pathogens (Cheroutre and Madakamutil, 2005). However, studies from our laboratory have found that cervical CD4 cells are also significantly more activated than blood CD4 T cells (Jaspan *et al*. manuscript submitted). At mucosal effector sites, T cells are exposed to high levels of foreign antigens in an inflammatory environment which can be exacerbated during HIV infection (Hladik *et al*., 2007; Mehandru *et al*., 2004; Mattapallil *et al*., 2005;

Nkwanyana *et al*., 2009). In HIV-infected individuals, CD4 TEM that traffic to mucosal effector sites may be continuously activated, infected by HIV and/or killed by activation induced apoptosis at mucosal sites preventing an effective immune response (Centlivre *et al*., 2007). Despite the high frequencies of CD4 TEM cells at the cervix, the fact that they are also highly activated suggests that they would be likely to be apoptotic and therefore less responsive in HIV-infected women compared to uninfected women. These studies suggest that T cells from cervical compartment may be pre-activated *in vivo* (before sampling) making them prone to activation-induced cell death upon *in vitro* stimulation of genital T cells, possibly explaining their lack of responsiveness *in vitro.* The inclusion of an apoptosis marker in the flow cytometry panel could have provided direct information on this possibility. The *in vitro* signals involved in T-cell differentiation and apoptosis may, however, differ from the *in vivo* environment.

The use of PMA/ionomycin-specific T cell responses allowed evaluation of the impact of HIV infection on the innate capacity of T cells to respond (global responsiveness). However, it is not clear whether PMA/ionomycin is relevant *in vivo* compared to the relevance of HIV-specific response. Nevertheless, PMA/ionomycin made it possible to conduct comparisons based on satus and moreover PMA/ionomycin has the advantage of activating sufficient proportions of rare T cells to give information about cytokine signature of each subset (Kim *et al*., 2009).

HIV-specific responses were rare with the exception of IFN-γ production by blood CD8 TEM and TEff cells. IL-2 secreting T cells, though very rare, were mainly CD8 TCM and CD4 TCM/TEM cells. TCM cells are thought to be responsible for the long-term maintenance of immune memory because they are able to traffic to secondary lymphoid organs and produce high levels of IL-2 (Lanzavecchia and Sallusto, 2005). Their ability to produce IL-2 has been suggested to prompt TCM cells to proliferate quickly and thus self-renew (Lanzavecchia and Sallusto, 2005). In contrast, TEM cells, that are predominantly located at the effector sites, are likely to produce both IFN-γ and IL-2 but lack the ability to self-renew (Harari *et al.*, 2004; Riou *et al.*, 2007; Tilton *et al.*, 2007). The frequency of HIV-specific T cell responses in this study was lower than that from previously published frequencies of Gag-specific responses at the cervix of HIV-infected women. This may be partially due to the fact that the donors used in this study were not pre-screened to select for women with high frequency

Gag-specific responses in blood as has been done in other similar studies where donors were pre-selected based on blood HIV epitope mapping (Kaul *et al*., 2000; 2003). Furthermore, this study only evaluated two cytokines (IFN-γ and IL-2) and these are unlikely to comprehensively represent the cytokines secreted by late memory subsets that pre-dominated at the cervix. In agreement with this, Kim *et al*. (2009) recently showed that late memory CD8 T cells stimulated with PMA/ionomycin and cytomegalovirus antigen predominantly produced CC chemokines (MIP-1β, MIP-1α and RANTES) but not IL-2.

In contrast to cervical responses to HIV Gag, HIV-1 specific CD8 and CD4 T cell responses to Gag in blood almost exclusively produced IFN-γ. These data suggest an altered response during HIV infection of which HIV may directly or indirectly trigger T cells to predominantly secrete IFN-γ rather than IL-2. High frequencies of HIV-specific IL-2 and dual IL-2/IFN-γ secreting CD4 T cells have been shown to be associated with better control of viremia during HIV infection (McNeil *et al*., 2001; Iyasere *et al*., 2003; Younes *et al*., 2003; Harari *et al*., 2004; Potter *et al*.,2007). Younes *et al.* (2003) found that individuals with persistent viremia had CD4 TCM cells lacking the ability to produce IL-2 and CD4 TEM cells that produced only IFN-γ. The exact mechanism by which HIV alters the ability of T cells to produce IL-2 and suppresses proliferation of HIV-specific CD4 and CD8 T cells remains to be determined.

Champagne *et al*. (2001) has previously shown that long term *in vitro* stimulation (36 hours) of memory T cells caused phenotypic and maturational changes in HIV-tetramer positive cells that were not observed in tetramer negative populations. In this study, activation-induced T cell maturation is unlikely to have played a role since the duration of stimulation with both PMA/ionomycin and HIV Gag peptides was short (4 hours and 6 hours, respectively) and the memory phenotype of CD4 and CD8 T cells stimulated with PMA/ionomycin or Gag was similar to the phenotype of CD4 and CD8 T cells that were not stimulated (data not shown). Although previous studies found an association between the memory phenotype of HIV-specific T cell and their ability to control HIV (Addo *et al*., 2007; Northfield *et al*., 2007, Akondy *et al*., 2009; Burgers *et al*., 2009), the number of women included in this chapter that responded to Gag made a similar analysis impossible to perform.

In conclusion, a significantly altered functional profile during chronic HIV infection has been demonstrated in polyclonaly-stimulated CD4 TEM cells in the female genital tract. I hypothesize that cervical CD4 TEM cells during HIV infection display a high level of pre-activation *in vivo* which predisposes them to apoptosis upon stimulation *ex vivo*. High level of immune activation and progressively declining CD4 T cells at mucosal sites, might account for the observed cervical CD4 dysfunction during HIV infection. These results also imply that mucosal memory T cells initially supplied by blood-borne or re-circulating memory T cells from other tertiary tissue may undergo selective modifications upon entering mucosal peripheral tissues due to local factors present in the mucosal microenvironment. Such mucosal factors can play an important role in determining the phenotype and fate of the responder cells. The local mucosal environment should be considered when the quality and quantity of mucosal T cells in mucosal HIV vaccine studies are tested.

Chapter 5

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Chapter 5
Role of inflammation in differentiation and homeostasis of T cells in the female genital tract

5.1 Summary

HIV infection is characterized by generalized high levels of immune activation that plays an important role in the depletion of CD4 T cells. The precise mechanisms leading to recruitment and activation of immune cells into the female genital tract are not clear. The aim of this study was to investigate the role of inflammatory and homeostatic cytokines present in the genital tract on the differentiation status of cervical T cells and maintenance of mucosal CD4 T cell numbers. Concentrations of inflammatory (IL-8, IL-6 and IL-1β) and homeostatic cytokines (IL-7 and IL-15) were measured in genital secretions by ELISA and compared with differentiation status of cervical T cells (defined according to expression of CD45RA, CD27 and CCR7). Both inflammatory (IL-1β) and homeostatic (IL-7) cytokine concentrations were significantly higher in HIV-infected women compared to uninfected women ($p=0.02$ and $p=0.04$, respectively). The concentration of IL-7 was associated with the concentration of inflammatory cytokines IL-6 ($p=0.005$, $Rho=0.46$); IL-1β ($p=0.02$, $Rho=0.40$); and IL-8 ($p=0.003$, $Rho=0.48$) in all women irrespective of their HIV status, suggesting involvement of IL-7 in the regulation of inflammation. Furthermore, genital tract concentrations of IL-7 were negatively associated with the frequency of cervical CD4 T cells, irrespective of HIV status ($Rho=-0.39$; $p=0.02$), suggesting that local IL-7 concentrations increase in response to depletion of cervical CD4 T cells. Overall, these data suggest a role for IL-7 in the maintenance of CD4 T-cell upon depletion in an inflammatory environment that may favour CD4 depletion.

5.2 Introduction

Infection with HIV is characterized by a progressive decline in CD4 T cell numbers and loss of T cell function (Sousa *et al*., 2002; Anthony *et al*., 2003). Mucosal surfaces of the body serve as a hot spot for CD4 T cell depletion during HIV infection (Li *et al*., 2005; Johnson and Kaur, 2005; Mattapallil *et al*., 2005). Chronic immune activation is a major contributor to the pathogenesis of HIV (Brenchley *et al*., 2006; Appay and Sauce 2008). Despite the link between T cell activation and HIV disease progression, the mechanism/s by which immune activation results in the CD4 depletion are still unclear (Boasso and Shearer, 2008; Sedaghat *et al*., 2008).

During acute HIV and SIV infections, CD4 TEM cells are rapidly depleted at effector sites while the TCM pool is preserved (Picker *et al.*, 2004; Grossman *et al.*, 2006; Picker, 2006). During chronic HIV infection, TEM cells are dependent on TCM cells for their homeostasis and this TCM-derived TEM production progressively declines during chronic infection (Marzo *et al.*, 2007; Okoye *et al.*, 2007).

Local factors in the cervical microenvironment such as inflammation and common cytokine receptor γ-chain family cytokines may have an impact on T cell distribution and differentiation profiles in the genital mucosa. Pro-inflammatory cytokines such as IL-1β and IL-6 in the genital tract have been associated with recruitment, differentiation and activation of immune cells (Belec *et al*., 1995; Sonza *et al*., 1996; Crowley-Nowick *et al*, 2000; Nkwanyana *et al*., 2009). Pro-inflammatory cytokines can also drive T helper (Th)0 cells to differentiate along the Th1 pathway (Crowley-Nowick *et al*., 2000). Furthermore, Geginat *et al*. (2001) showed that *in vitro* addition of cytokines TNF-α, IL-6, IL-10 and IL-12 resulted in TCM cells differentiating into TEM-like cells expressing the CCR5 chemokine receptor.

Common-γ_c family cytokines such as IL-7 and IL-15 play a role in the development, proliferation, survival and differentiation of multiple cell lineages of both innate and adaptive immunity (Sallusto *et al*., 2004; Rochman *et al*., 2009). Unutmaz *et al.* (1994; 1995) demonstrated that T cells derived from blood may proliferate *in vitro* in an antigen-independent manner in the presence of these common γc receptor binding cytokines (IL-2, IL-4, IL-7 and IL-15). IL-15 plays a major role in the homeostatic regulation and renewal of

memory T cells (Tan *et al*., 2002). Picker *et al* (2006) showed that IL-15 induces CD4 TEM cell production and tissue emigration in non-human primates.

Cytokines play an important role in HIV-1 infection. IL-7 contributes to the development, proliferation, and homeostatic maintenance of T cells (Grabstein *et al*., 1990; Schluns *et al*., 2000; Fry *et al*., 2001; Llano *et al*., 2001; Tan *et al*., 2001). T-cell depletion is associated with increases in circulating IL-7 levels, suggesting that IL-7 may be involved in homeostatic regulation of T-cell numbers and restoration of host immune competence. (Fry *et al*., 2001; Llano *et al*., 2001, Napolitano *et al*., 2001). The role of IL-7 as a central regulator of the survival and homeostasis of CD4 and CD8 T cells has been well established in systemic studies (Llano *et al*., 2001; Napolitano *et al*., 2001), but whether this also applies to T cells in the mucosal compartments has not been studied. Ironically, high levels of IL-7-driven T-cell proliferation during HIV infection can exacerbate viral replication. Not only will HIV replicate more efficiently in dividing cells, but IL-7 also acts as a co-factor in the transactivation of the long terminal repeat of HIV (Uittenbogaart *et al*., 2000; Weitzmann *et al*., 2000; McCune, 2001; Chomont *et al*., 2009).

In this Chapter, the role of IL-7 and IL-15 in the survival and homeostasis of cervical T cells and how that role is regulated by other local mucosal factors was investigated. Investigating the role of inflammatory and homeostatic cytokines in the modulation of the memory T cell pool during HIV infection may shed light on events that lead to local CD4 T cell depletion during HIV infection women.

5.3 Methods

5.3.1 Description of individuals included in the study

Twelve chronically HIV-infected and 36 uninfected women attending the Emphilisweni community clinic in Gugulethu, Cape Town were enrolled in this study (described in Chapter 3 and 4). Women who were menstruating at the time of sampling, who were post-menopausal, had undergone a hysterectomy, had vaginal discharge, or visible or reported STIs were excluded from the study. The study was approved by the Health Sciences

Research Ethics Committee of the University of Cape Town, South Africa (UCT REC 258/2006) and informed written consent was obtained from all women in the study.

5.3.2 Cervical sample collection and processing

Cervical cells were collected and isolated as described in Chapter 2 section 2.3.2. Cervical cells were pelleted and counted while the 3ml transport media supernatant fraction from flushing the cytobrush was collected and frozen. The supernatant from flushing the cytobrush was stored for evaluation of HIV shedding in the genital tract and measurement of cytokines.

5.3.3 PBMC isolation

Blood was collected and processed as described in Chapter 2 section 2.3.5.

5.3.4 Counting of cervical and blood mononuclear cells

Cervical CD3+ cell numbers were evaluated by Guava automated cell counting (Guava technologies, Hayward, CA) as described previously in Chapter 2 section 2.3.4.

5.3.5 Flow cytometry

Cervical and blood cells were evaluated for cell surface expression of maturational markers CD45RA, CD27 and CCR7 as previously described in Chapter 3 section 3.3.6. FlowJo software (Tree Star) was used for colour compensation and data analysis. Fluorescence minus one (FMOs) were used to set gates.

5.3.6 Measurement of cytokine concentrations in genital secretions

Concentrations of IL-1β, IL-6, IL-8, IL-15 and IL-7 in cervical supernatants were determined using Quantikine high sensitivity (IL-1β, IL-6, IL-8, IL-7) and Quantiglo chemiluminescent (IL-15) ELISA kits (R&D Systems Inc., Minneapolis, MN) according to manufacturer's instructions. The limit of detection of IL-1β, IL-6, IL-8, IL-7, and IL-15 assays was ≥1 pg/ml, 0.7 pg/ml, 1.5 pg/ml, 0.1 pg/ml and 0.1 pg/ml, respectively. All standards were run in duplicate while cytokine levels in cervical supernatants were measured in singlet. Cervical supernatants were assayed neat for IL-1β, IL-6, IL-15 and IL-7. Cervical supernatants were

diluted 1:20 using the calibrator diluent (R&D Systems Inc., Minneapolis, MN) for measurement of IL-8. ELISA plates were read on a VersaMax® ELISA microplate reader and data analysed using SoftMax Pro Ver. 4.3.1 Software (Molecular Devices).

5.3.7 Determination of viral load in cervical supernatant and plasma

HIV load was determined in cervical and plasma samples using Nuclisens Easyq HIV 1 Version 1.2 by the NHLS Diagnostic Virology Laboratory (Groote Schuur Hospital, Cape Town, South Africa). The detection limit of this assay is ≥50 HIV RNA copies/ml and values that were below the assay's detection limit were reported as zero.

5.3.8 Statistical analysis

Mann–Whitney U and Student's unpaired T tests were performed for independent sample comparisons and Spearman Rank tests were applied for correlations, using GraphPad Prism version 5.0. All tests were two-tailed and $p \leq 0.05$ were considered significant.

5.4 Results

Twelve HIV-infected and 36 uninfected women were included in this study to determine the impact of local factors (inflammation and common-γ_c family cytokines) on cervical T cell differentiation profiles. The clinical details of women studied have been previously described in Chapter 3 Section 3.4 Table 3.1. The median age of HIV-infected women was 35.0 years (IQR 28-43) while uninfected women were a median of 35.5 years (IQR 30-47). In HIV infected women, the median blood CD4 T cell count was 292 cell/µl (IQR 142-559) and median HIV viral load in plasma was 3000 RNA copies/ml (IQR 180-31000). The median HIV viral load detected in cervical secretions was 200 RNA copies/ml (IQR 0-540). The cervical CD4:CD8 ratio was significantly lower in HIV-infected women than uninfected women [median 0.58 (IQR 0.29-0.85) for HIV+ versus 2.17 (IQR 1.29-3.44) for HIV- women; $p<0.0001$] suggesting that the proportion of CD4 T cells were significantly reduced in HIV-infected compared to uninfected women. All women included were naive to anti-retroviral therapy at the time of study.

5.4.1 Comparison of inflammatory and homeostatic cytokine concentrations in the female genital tract of HIV-infected and uninfected women

The concentration of inflammatory cytokines IL-6, IL-1β and IL-8 and homeostatic cytokines IL-7 and IL-15 were measured in cervical secretions from HIV-infected and uninfected women (Figure 5.1A-E). IL-6, IL-1β and IL-8 were measured in this Chapter because these cytokines were more readily detectable at the cervix compared to other inflammatory cytokines (TNF-α, IL-12 and IL-10) measured in Chapter 2. IL-8 was present at the highest concentrations in cervical secretions of the cytokines measured. In contrast, IL-15 in genital secretions was generally present at concentrations at the detection limit of the assay, irrespective of HIV status. HIV-infected women had significantly higher concentrations of inflammatory IL-1β and homeostatic IL-7 in their genital secretions compared with HIV uninfected women (p=0.02 and p=0.04; Figure 5.1B and D respectively).

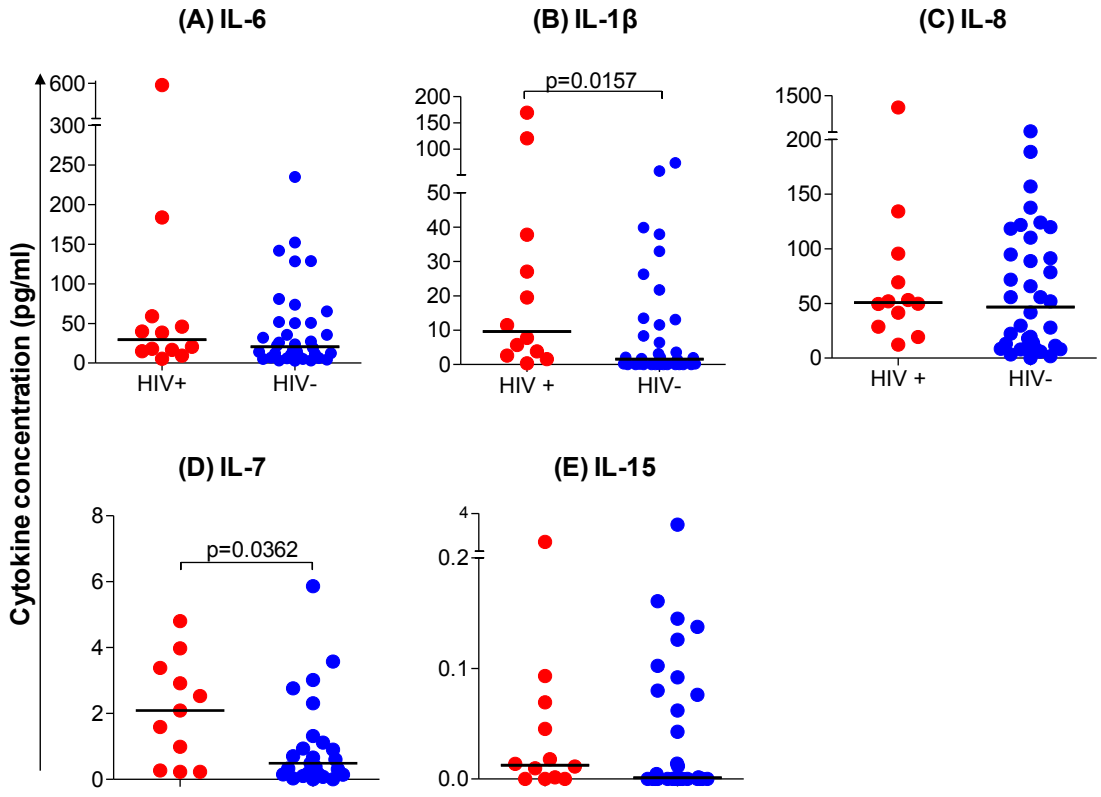

Figure 5.1 Comparison between inflammatory (IL-6, IL-1β and IL-8) and homeostatic cytokine (IL-7 and IL-15) concentrations in cervical supernatants of HIV-infected and uninfected women. Concentrations of inflammatory cytokines (A) IL-6, (B) IL-1β and (C) IL-8 and homeostatic cytokines (D) IL-7 and (E) IL-15 in cervical supernatants from HIV-infected (red circles) and HIV uninfected (blue circles) women were measured. The horizontal line indicates the median value for each group. Differences between groups were calculated using Mann-Whitney U test. P-values <0.05 were considered significant.

5.4.2 Relationship between inflammatory and homeostatic cytokines in the genital tract

Previous studies have described the role of IL-7 contributing to a vicious cycle promoting inflammation and/or immune activation (Roato *et al*., 2006; Churchman and Ponchel 2008). IL-1β and TNF-α have been shown to increase the stromal production of IL-7 which, in turn, up-regulates the production of TNF-α by macrophages (Harada *et al*., 1999). The association between genital tract inflammation and IL-7 was investigated (Figure 5.2). The concentration of IL-7 at the cervix was found to be positively associated with the concentrations of inflammatory cytokines IL-6 (Rho=0.46; p=0.005; Figure 5.2A), IL-8 (Rho=0.48; p=0.003; Figure 5.2B) and IL-1β (Rho=0.40, p=0.02; Figure 5.2C), irrespective of HIV status.

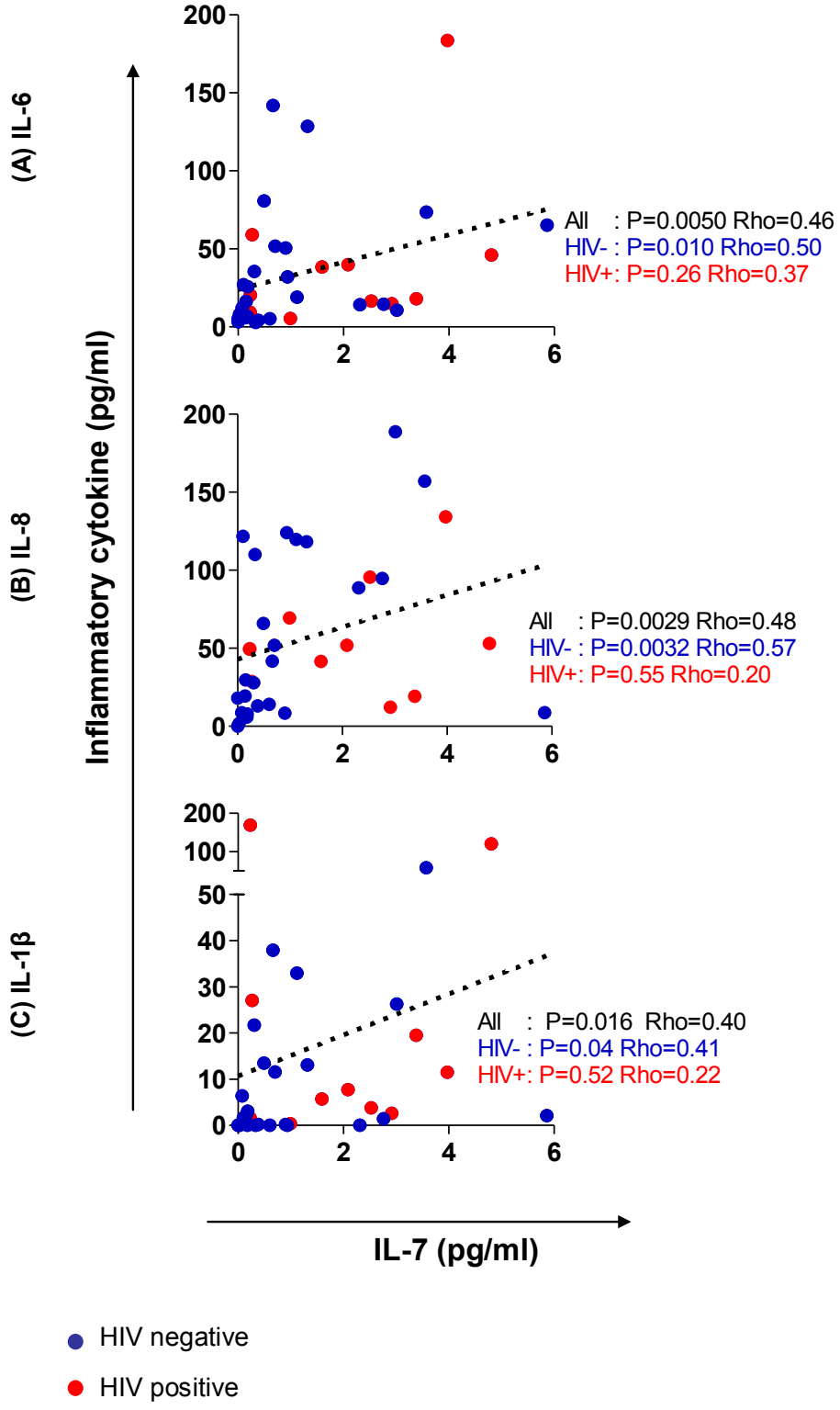

Figure 5.2 Association between the concentration of IL-7 in the female genital tract and genital inflammation. Correlation between IL-7 and the inflammatory cytokines (IL-6, IL-8 and IL-1β) levels detected per participant in cervical supernatants. Each dot represents an individual donor, HIV negative (blue dots) and HIV positive (red dots). The line is the linear regression curve. P-values were calculated using Spearman rank test and p values ≤0.05 were considered significant.

5.4.3 Association between IL-7 and CD4 T cell numbers in the female genital tract

In Chapter 3, the frequency of CD4 T cells at the cervix and in blood were significantly reduced in HIV-infected compared to uninfected women and that the extent of CD4 depletion in blood was mirrored at the cervix (Section 3.4.2 Figure 3.1). Previous studies have found an inverse correlation between plasma IL-7 concentrations and CD4 counts in blood of HIV-infected individuals (Llano *et al.*, 2001; Napolitano *et al.*, 2001; Albuquerque *et al.*, 2007) which suggests a possible mechanism for the increased homeostatic expansion observed in CD4 depleted individuals. To determine whether IL-7 plays a similar role in the regulation of CD4 T cells in the female genital tract, the relationship between the genital IL-7 and frequency of cervical CD4 T cells was determined (Figure 5.3A). An inverse relationship was observed between the frequency of cervical CD4 T cells and cervical IL-7 concentrations in all women (p=0.02, Rho=-0.39; Figure 5.3). These data suggest that local IL-7 plays a role in maintenance of CD4 T cell homeostasis in the female genital tract upon CD4 depletion.

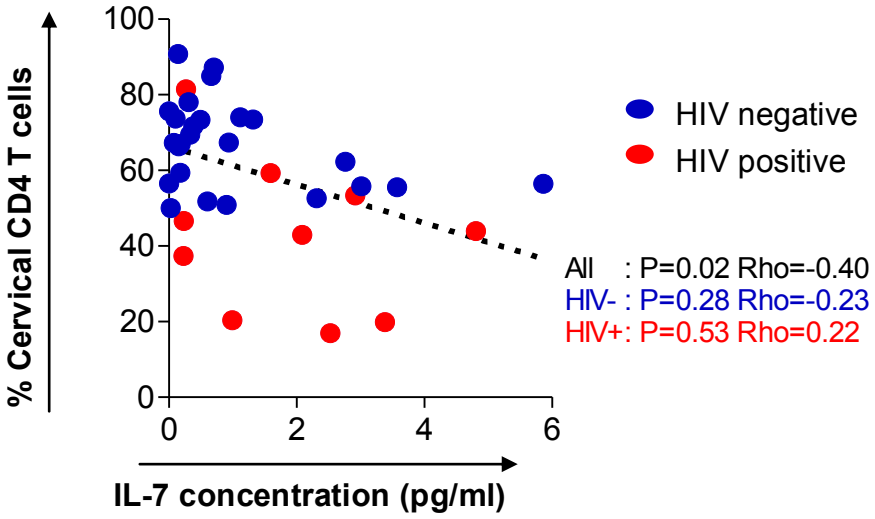

Figure 5.3 Association between CD4 counts and IL-7 concentrations in the female genital tract. Relationship between IL-7 concentrations in the female genital tract and (A) the frequency of cervical CD4 T cells, irrespective of HIV status. IL-7 concentrations were measured in cervical supernatants from HIV-infected women and HIV uninfected. Each dot represents an individual patient. Spearman rank test was used to test the association and $p<0.05$ were considered significant.

5.4.4 Association of genital tract cytokine concentrations and T cells differentiation status in the genital mucosa

Previous *in vitro* studies have shown that proliferation in the presence of cytokines (TNF-α, IL-6, IL-10 and IL-12) impacts on T cell differentiation status (Geginat *et al*., 2001). It was hypothesized that the presence of high levels of inflammatory cytokines, such as during HIV infection, these cytokines would stimulate T cells to proliferate and significantly alter the differentiation status of T cells in the genital tract. To investigate the impact of genital inflammation on the differentiation status of T cells in the female genital tract, the distribution of distinct memory subsets at the cervix were compared to the concentrations of IL-6, IL-8 and IL-1β detected in genital secretions.

No association was observed between genital tract inflammation and T cell memory subset distribution in HIV-infected women (data not shown). The presence of HIV may result in chronic antigenic stimulation of cervical T cells making it difficult to detect the direct effect of inflammation on the differentiation status of cervical T cells. Whether these associations would gain statistical significance in the case of the larger sample size is not known.

Despite high concentrations of IL-7 in genital secretions being associated with reduced frequencies of CD4 T cells at the cervix (section 5.4.3), no association was found between cervical IL-7 concentrations and frequencies of distinct T cell differentiation subsets in either uninfected or HIV-infected women (data not shown).

5.5 Discussion

Previous studies have reported increased concentrations of IL-7 in individuals with low CD4 counts associated with HIV infection or depleted T cell numbers as a result of chemotherapy for cancer (Fry *et al*., 2001; Llano *et al*., 2001; Napolitano *et al*., 2001). The results presented in this study suggest that in the female genital tract microenvironment of HIV-infected women IL-7 levels are increased and there is an also high concentration of inflammatory cytokines. Increased concentrations of cervical IL-7 were observed in women with low frequencies of cervical CD4 T cells. IL-7 in the female genital tract is thought to be involved in the cervical CD4 T homeostasis upon CD4 depletion. Furthermore, genital tract concentrations of inflammatory IL-6, IL-1β and IL-8 were associated with the concentrations of IL-7 in all women. These results implicate inflammation in promoting an activated environment that may favour CD4 depletion and increased concentrations of IL-7 in the presence of HIV.

Since IL-7 has been shown to be the central regulator of peripheral T-cell homeostasis (Fry *et al*., 2003), it has been hypothesized that IL-7 therapy will potently immunomodulate both T-cell–depleted and T-cell–replete humans. There are ongoing trials of IL-7 supplementation to HIV-infected individuals showing that IL-7 administration drives T cell cycle entry and expansion in HIV-1 infection (Sereti *et al*., 2009). IL-7 therapy in normal and simian immunodeficiency virus (SIV)–infected nonhuman primates did not induced up-regulation of classical activation markers such as CD69, HLA-DR, or CD25; however, Fas (or CD95) a death receptor, was up-regulated on both CD4 and CD8 T cells (Fry *et al*., 2003).

Despite the promising features of IL-7 for its potential use in HIV infection, the observation that during HIV infection high levels of IL-7 are correlated with CD4 T depletion and appear not to be beneficial to rescue the diminishing T cell pool, has led to some *in vitro* studies suggesting an alternative hypothesis. These studies have predicted that the elevated IL-7 may contribute to HIV associated immune pathophysiology (Capitini *et al*., 2009). IL-7 promotes HIV replication *in vitro*, as has been shown in thymic organ cultures (Guillemard *et al*., 2001; Pedroza-Martins *et al*., 2002) and IL-7 also enhances HIV entry into cells through CXCR4 (Pedroza-Martins *et al*., 1998). IL-7 has been shown to induce activation of monocytes and

NK cells, leading to enhanced production of inflammatory cytokines (Harada *et al*., 1999; Li *et al*., 2000; Dåmas *et al*., 2003; Churchman and Ponchel 2008). High levels of IL-7 also up-regulate Fas expression on naı¨ve T cells, and promote Fas-induced apoptosis in T-cell cultures infected with HIV, suggesting that Fas pathway may play a role in depletion of T cells during HIV infection (Fluur *et al*., 2007). It is not known whether these *in vitro* observations will be relevant *in vivo*.

In this study cervical IL-7 levels in HIV-uninfected women are positively associated with IL-1β, IL-6 and IL-8 levels. Whether these associations would gain statistical significance in HIV-infected women in case of a larger cohort size is not known. This study cannot establish the cause and effect (if any) between inflammation and IL-7. The difficult in interpretation of this data may be because it was not the major aim of this study to characterise the cytokine pattern in relation to vaginal infections which are prevalent during HIV infection. Indeed, bacterial vaginosis has been clearly associated with high levels of IL-1β or TNF-α (Sturm-Ramirez *et al*., 2000). Studies done in our laboratory have shown increased concentrations of pro-inflammatory cytokines such as IL-6 and IL-1β in the female genital tract of acutely and chronically HIV-infected women compared to uninfected women (Bebell *et al*., 2008; Nkwanyana *et al*., 2009). Previous studies have proposed that both direct (HIV) and indirect (increased susceptibility to sexually transmitted infections) may be the cause of chronic immune activation and inflammation during chronic HIV infection (Moodley *et al*., 2002; Appay and Sauce, 2008; Mitchell *et al*., 2008). This suggests that cervical T cells are exposed to a significantly elevated inflammatory environment in the female genital tract that is both directly and indirectly influenced by the virus during HIV infection. This activated environment is likely to promote depletion of CD4 T cells particularly in HIV-infected women. CD4 proliferation assays in the response to IL-7 in the presence or absence of inflammatory cytokines in the future *in vitro* studies could directly address some of the observations noted here.

This study found that concentrations of IL-7 in the genital tract were associated with the extent of CD4 depletion at the cervix. Increased production of IL-7 could be a homeostatic response to CD4 depletion. Indeed, there were high levels of IL-7 in cervical secretions of HIV-infected women, who also happened to have high CD4 depletion. Previous studies in humans have confirmed that depletion of CD4 T cells was associated with increased

production of IL-7 (Fry and Mackall, 2005; Catalfamo *et al*., 2008; Rochman *et al*., 2009). In the presence of high levels of HIV viremia, previous studies have shown that T cells are more susceptible to loss of CD127 expression and as a result these T cells are unable to benefit from the growth promoting signals provided by IL-7 (Wherry *et al*., 2006; Mercier *et al*., 2008). Cervical T cells are constantly exposed to pathogens in the female genital tract, making them likely to remain activated. Li *et al*. (2003) showed that activated T cells express little or no CD127 but regain expression when they are rested *in vitro* or *in vivo*. The result of this being that activated T cells such as during chronic HIV infection have reduced ability to use IL-7. Because there was no significant association between cervical IL-7 levels and frequency of CD4 T cells when HIV-infected and uninfected women were separated, this finding should be interpreted with caution. The inverse relationship of cervical IL-7 with the frequency of CD4 T cells was only significant when HIV-uninfected and infected women were pooled, with CD4 T cell values for HIV-infected women falling in a lower strata than those of uninfected women.

T cells can expand and differentiate in response to stimulation by cytokines and chemokines (Sinigaglia and D´ambrosio, 2000). This study did not find an association between inflammatory cytokine concentrations in the genital tract of HIV-infected women and differentiation of cervical T cell subsets. This association may have been masked directly by HIV, which contributes to activation and loss of T cells (Merrill *et al*.,1989; Rieckmann *et al.,* 1991; Lee *et al*., 2003). Furthermore, there are a few drawbacks to this study. Given that women included in this Chapter were only syndromically screened for STIs and the fact that STIs also have an association with HIV shedding, inflammation, and genital immunity (Rebbapragada *et al*., 2007; 2008), this could limit the ability to clearly establish cause and effect between HIV, inflammation and differentiation status of cervical T cells.

IL-7 is thought to be involved in homeostasis of both naïve and memory T cells (Fry and Mackal, 2005; Sieg *et al*., 2005) while IL-15 is mainly involved in homeostatic proliferation of memory T cells as well as antigen-independent proliferation and differentiation of TCM cells to TEM cells (Geginat *et al*., 2003; Picker *et al*., 2006; Purton *et al*., 2007; Rochman *et al*., 2009). Despite the potential role of IL-7 in genital tract CD4 reconstitution, no relationship was found between IL-7 concentrations and specific memory T cell subsets. Previous studies have shown that IL-7 supports the survival of IL-7 receptor (CD127)-bearing T cells at lower concentrations of IL-7 (Li *et al*., 2003; Lenz *et al*., 2004). Since

majority of cervical T cells exhibit a TEM phenotype, a phenotype that has been shown to lack IL-7 receptor expression in blood (Burgers *et al*., 2009), this could partially explain lack of association observed. Because low cervical cell numbers result in only very few of the low frequency T cell subsets being available for analysis (Chapter 3), it would be interesting in future studies to use vaginal biopsies rather, where T cell numbers of the less frequent subsets would not be as limiting. Previous studies have shown that IL-7 at elevated concentrations induces cell division (Li *et al*., 2003; Lenz *et al*., 2004). In future *in vitro* studies distinct memory T cell phenotypes could be sorted, to test whether addition of exogenous IL-7 induced expression of the Ki67 proliferation marker and CD127. These future studies would be useful in determining the subset that is most responsive to IL-7 and the concentration of IL-7 at which the IL-7 responsive T subsets would start to proliferate.

This study found negligible amounts of IL-15 in the genital secretions of HIV-infected and uninfected women. This may be due to the fact that cervical mucosa is a site that has heightened antigenic stimulation in which differentiation of TCM cells to TEM cells is predominantly carried out in an antigen-dependent manner. As a consequence, IL-15-mediated antigen-independent development of TEM cells may be inhibited or limited in the female genital tract.

In conclusion, future mechanistic studies are needed to establish whether the association of IL-7 with inflammatory cytokines observed in this preliminary study are as a result of the direct association between IL-7 and inflammation as has been suggested by other previous *in vitro* studies (Harada *et al*., 1999; Li *et al*., 2000; Churchman and Ponchel 2008) or whether inflammation and immune activation promote CD4 depletion, indirectly leading to increased IL-7 levels as a homeostatic response to CD4 depletion (Fry *et al*., 2001; Llano *et al*., 2001; Napolitano *et al*., 2001; Fry *et al*., 2003). The latter proposition is likely to be true. This could be tested by measuring cervical CD4 proliferation in response to IL-7 in the presence and absence of inflammatory cytokines. IL-7 supplementation in HIV infected will probably be more effective in CD4 restoration when inflammation (activation) is controlled.

Chapter 6
Conclusions and General Discussion

The female genital tract plays an important role in HIV acquisition and transmission during heterosexual contact. Knowledge of cervicovaginal environment and its role in HIV pathogenesis is therefore important for understanding of the mechanisms behind ultimate immune system failure and effective vaccine design. A number of studies have highlighted the importance of the mucosa in HIV pathogenesis and HIV is now increasingly being recognized as a disease of the mucosal immune system (Musey *et al*., 2003a; Johnson and Kaur, 2005; Li *et al*., 2005; Mattapallil *et al*., 2005). HIV-specific T cell responses are essential in the control of HIV as they exert pressure on the virus, causing it to escape (Peut and Kent, 2006). However, we do not fully understand how HIV causes infection and disease. Early in the epidemic, studies suggested a simple model which predicted that HIV-mediated destruction of CD4 T cells directly reduced the number of these cells with the body slowly struggling to replace these losses with new cells as the disease progresses (Ho *et al*., 1995; Wei, *et al*. 1995; Mohri *et al*., 1998). However, it has recently emerged that immune activation contributes significantly to CD4 T cell loss and HIV pathogenesis (Sousa *et al*., 2002; Hazenberg *et al*., 2003; Deeks *et al*.,2004; Kornfeld *et al*., 2005; Brenchley *et al*., 2006; Appay and Sauce, 2008).

Although immune activation in response to invading pathogens is a crucial component of host protective immunity, such responses may ironically also contribute to HIV pathogenesis by providing the virus with a steady supply of activated susceptible target cells (Lawn *et al*., 2001). In this thesis, I investigated the interplay between mucosal HIV-specific-CD8 T cells, HIV shedding and inflammation localized in the cervical micro environment in women chronically infected with HIV. This study showed that cervical environment is defined by high levels of non-specific activation (indicated by high levels of pro-inflammatory cytokines). HIV-specific CD8 T cells at the cervix were detectable, as has been previously shown by others (Musey *et al*., 1997; Kaul *et al*., 2003; Musey *et al*., 2003a; 2003b). There was no significant correlation between the magnitude of HIV-specific responses in blood and

those detected at the cervix suggesting that systemic immune responses, currently assayed in vaccine trials, may not be an appropriate predictor of *ex vivo* responses at mucosal surfaces where HIV acquisition occurs. Furthermore, there was no significant correlation between cervical T cell responses and HIV shedding. However, there was a positive association between the magnitude of HIV-specific cervical T cell responses and genital tract inflammation. Women who were shedding HIV had significantly higher levels of inflammation compared to women who were not shedding. While the cause and effect relationship is not clear, these results suggest that inflammation and immune activation may drive local shedding and HIV-specific CD8 T cell recruitment. These cervical HIV-specific T cells were, however, not able to control local HIV infection because they exist in an environment that promotes non-specific activation that is beneficial to HIV replication.

An additional level of complexity in HIV pathogenesis is related to the observation that not all CD4 T cells are equally targeted by the virus, and this may depend on the state of CD4 T-cell activation, differentiation and anatomic distribution (Grossman *et al.*, 2006). It has been shown that SIV selectively targets and destroys specific subsets of CD4 T cells that are abundant in mucosal tissues but rare in peripheral lymphoid tissues (Veazey *et al.*, 2000b). HIV and SIV entry is confined to intermediate and short-lived CD4 T cells which have high frequencies of CCR5 expression compared to naive and central memory CD4 T cells that are by and large CCR5-negative (Grossman *et al.*, 2006). Since the differentiation status of T cells is known to play an important role in HIV pathogenesis, I characterized T cells in the genital tract in the absence of HIV infection and during chronic HIV infection by differential staining for CD45RA, CD27 and CCR7. This study showed that HIV-infected women have significantly reduced frequencies of CD4 T cells compared to uninfected women in both blood and at the genital tract. The majority of T cells in the female genital tract were TEM in phenotype. Furthermore, HIV-infected women had significantly high levels of terminally-differentiated CD8 and CD4 T cells compared to uninfected women. In HIV-infected women, there was no association between differentiation status of cervical T cells and amount of HIV virus present in genital secretions. However, reduced frequencies of long-lived T cells (TCM) and high frequencies of intermediate (TInter) and short-lived (TEff) T cells, irrespective of HIV status, were associated with reduced frequencies of CD4 T cells in the cervix.

These results suggest that reduced frequencies of CD4 T cells in the genital tract are the result of accumulation of T cells that are activated and differentiating towards a short-lived phenotype and these cells are destined to die of activation-induced apoptosis, regardless of the HIV status of the women. Inclusion of cellular markers of T cell activation such as CD38, HLA-DR, CCR5 and Ki-67 in addition to differentiation markers would have directly addressed this hypothesis although not included in this study. These findings suggested an important link between the frequencies of CD4 T cells at the cervix and the differentiation status of T cells in the female genital tract.

One of the major pathophysiological implications of HIV-associated death of activated T cells is that it results in dysfunctional 'helper' T-cells (Sodoro and Silvestri, 2010). Lack of HIV control occurs despite high frequencies of HIV-specific IFN-γ secreting CD8 T cells (Betts *et al.*, 2001), suggesting that measuring IFN-γ production alone by CD8 T cells is not an accurate correlate of viral control (Harari *et al*, 2004; Pantaleo and Koup, 2004; Harari *et al*, 2006). The presence of HIV-specific CD8 T cells that can proliferate and secrete IL-2 has been associated with low levels of HIV antigen load (Harari *et al.*, 2004). Moreover, the differentiation status of T cells has also been shown to have an impact on both qualitative and quantitative differences in cytokine production (Sallusto *et al.*, 1999; Masopust *et al.*, 2001). Previous studies have shown that TCM cells have high proliferative capacity but reduced cytotoxicity whereas terminally-differentiated TEM cells are highly cytotoxic with low proliferative capacity (Sallusto *et al.*, 1999; Younes *et al.*, 2003; Halwani *et al.*, 2006). In this study, the impact of T cell maturational status on the functional ability of T cells in the female genital tract was determined. I found that CD4 TCM cells mainly produced IL-2 whereas CD4 TEM produced both IFN-γ and IL-2 after polyclonal stimulation with PMA/ionomycin. It was found that CD8 T cells from HIV-infected and uninfected women that were stimulated with PMA/ionomycin had similar ability to secrete IFN-γ and IL-2. Conversely, CD4 TEM cells at the cervix of HIV-infected women had significantly reduced ability to produce IFN-γ and IL-2 compared to uninfected women upon polyclonal stimulation, suggesting that bulk CD4 TEM cells in the female genital tract are dysfunctional in HIV-infected women.

HIV-specific T cells that secreted IFN-γ were less frequently detected at the cervix compared to blood in HIV-infected women, making it difficult to evaluate the role of genital tract HIV-specific T cells in controlling HIV locally. The finding that impaired *in vitro* production of IFN-γ and IL-2 by cervical CD4 TEM cells (following PMA/ionomycin stimulation) in HIV-infected compared to uninfected women most likely indicates that TEM cells in HIV-infected women are highly activated, making them prone to activation induced cell death and less responsive when stimulated *in vitro*. Since several studies have suggested that the differentiation pathway from TEM to TCM is likely to be bi-directional (Sallusto *et al*., 1999; Wherry *et al*., 2003), the rate of transition from TCM to TEM phenotype is likely to increase in HIV-infected individuals as TEM cells get lost through activation-induced cell death, inevitably resulting in the loss of TCM pool that is meant to replenish the TEM pool. This ultimately results in the loss of CD4 T helper function and effective immune response that is characteristic of chronic HIV infection. The inclusion of an apoptotic marker in the flow cytometry panel could have strengthened this study.

The findings presented in Chapters 2, 3, and 4 of this dissertation all suggest that "immune activation" in the female genital tract rather than cytopathic properties of HIV account for the progressive immunodeficiency and ultimate immune collapse at this local site of infection and transmission, as suggested by Sodora and Silvestri (2008). The causes of the HIV-associated immune activation are still being debated. It has not been established whether the high T cell turnover observed in chronic HIV infection is caused by homeostatic proliferative response to ongoing HIV-mediated destruction of cells, persistent immune responses to ongoing antigenic stimulation, and/or proinflammatory signals (Jameson, 2002; Silvestri and Feinberg, 2003). In Chapter 5, the role of inflammatory and homeostatic cytokines in the recruitment and regulation of cervical T subsets was investigated. Elucidation of the mechanisms underlying the recurrent recruitment and activation of mucosal T cells represent an essential step for designing interventions to fully restore functional immunity in HIV-infected individuals. The results presented in Chapter 5 showed that increased concentrations of IL-7 was associated with depletion of CD4 T cells, indicating that IL-7 in the female genital tract may play a role in homeostatic regulation CD4 T cells upon CD4 depletion. Moreover, HIV-infected women (who were shown in Chapter 3

to have significantly depleted CD4 T cells) expressed significantly higher levels of IL-7 compared to uninfected women. Despite the observed role of IL-7 in genital tract CD4 restoration (Chapter 5), no relationship was found between the concentration of IL-7 in the genital tract and frequencies of any particular memory T cell subset. It will be important to determine in future *in vitro* studies which memory T cell subsets are induced to proliferate in the presence of exogenously added IL-7.

Interestingly, genital tract concentrations of inflammatory IL-6, IL-1β and IL-8 were associated with the concentrations of IL-7 suggesting a direct relationship between genital tract inflammation and homeostasis. Although the cause and effect relationship between inflammation and IL-7 is not clear, one interpretation is that IL-7 is "called in" to repair or reverse the effects of local inflammation by reconstituting and maturing CD4 T cells lost during HIV infection. Alternative to this hypothesis, *in vitro* studies showed that IL-7 directly induced activation of monocytes and NK cells, leading to enhanced production of inflammatory cytokines (Alderson *et al*., 1991; Harada *et al*., 1999; Li *et al*., 2000; Fry *et al*., 2001; Churchman and Ponchel 2008). However, it is not known whether these *in vitro* studies are appropriate *in vivo*. The association between inflammation, IL-7 and CD4 T cells could be validated by comparing proliferation ability of CD4 T cells in response to IL-7, in the presence or absence of pro-inflammatory cytokines.

Despite the evidence that inflammatory cytokines (such as TNF-α, IL-6, IL-10 and IL-12) may alter the differentiation status of T cells in an antigen-independent manner (Sallusto *et al*., 1999; Geginat *et al*. 2001), the association between inflammatory cytokines and the differentiation of individual T cell subsets was not evident in this study. This may be partly due to the fact that antigenic burden *in vivo*, that may influence the activation and differentiation status of T cells, could not be taken into account in this study, particularly in HIV-infected women where HIV itself and other opportunistic genital tract infections may cause persistent stimulation and differentiation of genital cells.

From the findings presented in this study, I propose a vicious infectious cycle that exists in the female genital tract between local persistent HIV infection, reduced

frequencies of CD4 T cells, an IL-7 homeostatic response in the presence of genital inflammation.

There are some limitations that need to be considered in the studies described in this dissertation. Studies discussed were largely of a descriptive nature with no mechanisms directly investigated and addressed. The lack of thorough screening for other asymptomatic STIs limited the interpretation of the findings in these studies, since STIs also have dramatic effects on HIV shedding, inflammation and genital immunology (Rebbapragada *et al*., 2007; 2008). It is not known whether some of the associations that were not observed in HIV-infected women were because of the lack of the statistical power due to a small sample size (Chapter 3, 4 and 5).

In summary, the studies described in this thesis give important insight into our understanding of HIV pathogenesis in cervical micro-environment. I showed that (i) genital tract inflammation promotes shedding of HIV virus in the female genital tract, and possibly also providing more targets (including virus-specific T cells) for the virus to replicate in; (ii) low frequencies of CD4 T cells in the female genital tract are associated with accumulation of short-lived T cells; (iii) CD4 TEM cells, a major subset of memory cells in the female genital tract, are dysfunctional during chronic HIV-infection; and (iv) a link exists between CD4 T-cell homeostasis involving IL-7 and inflammation. Altogether, this thesis confirms, extends and re-emphasizes that HIV is a disease of the mucosal immune system. Inflammation in the female genital tract needs be monitored with the hope that these studies will lead to identification of new therapeutic or prophylactic targets.

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